University of Alberta

Estrogenic endocrine disrupting compounds in Canadian wastewaters

by

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ABSTRACT

Several estrogenic endocrine disrupting chemicals (eEDCs) including industrial chemicals, natural and synthetic steroidal estrogens, and various naturally occurring phytoestrogens have been identified as priority organic pollutants with major source inputs being municipal wastewater treatment plant (WWTP) effluents. This research initially involved a survey of 5 treatment plants / processes in Western Canada (i.e. sites A, B, C, D and E) to provide information on the levels, profiles and total estrogenicity of wastewater effluents in the region by employing instrumental and bio-analytical methods. Several synthetic and natural steroidal estrogens along with estrogen analogues from industrial and domestic products were detected and prioritized from the surveyed wastewaters. Additionally, potential endocrine disruption effects were monitored in chinook salmon (*Oncorhynchus tshawytscha*) exposed to ecologically relevant concentrations (1%, 3% or 10%) from one of the plants assessed (site B). Despite no obvious biological effects being detected in the exposed salmon, linear relationships between estrogenicity predicted by chemical analysis and that measured via *in vitro* yeast assay (RYA) ($r = 0.73$-0.75; significant at 90-95% confidence) were observed for samples from site B and support the hypothesis that the targeted chemicals in this work were responsible for the bulk of the estrogenicity in domestic wastewater.

A week-long intensive sampling campaign was performed at site D (tertiary treatment) allowing for a detailed and accurate examination of the occurrence, in-

plant production and removal rates of key eEDCs at each treatment stage. The data obtained were fit to an existing mechanistic model to obtain kinetic and equilibrium constants for the natural steroidal estrogens known to pose the greatest risk to the reproductive health of aquatic organisms. Finally, temporal influent – effluent estrogenicity data was collected at site D from September to December and combined with operational, wastewater quality, and climate data to determine which of these variables may be related to the levels and reduction of RYA activity, intended as a surrogate measure of important eEDCs. No correlations were seen between RYA activity reduction and percent reduction in BOD, Flow, SRT or even rainfall. The reduction trends for RYA measured activity were explained best by an inverse relationship with ambient and effluent temperatures.

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List of Symbols and Abbreviations

Abbreviation	***Definition***
AL	Aerated lagoon
BKME	Bleached kraft mill effluent
BNR	Biological nutrient removal
BOD	Biochemical oxygen demand (Theoretical)
BOD$_5$	5-day biochemical oxygen demand
BPA	Bisphenol A
ASB	Aeration stabilization basin
AST	Activated sludge treatment
BSTFA	Bis-(trimethylsilyl)-trifluoroacetamide
C_s	Mass of substance sorbed to solids per unit reactor volume
$C_{s,floc}$	Mass of substance sorbed to solids per unit weight of solid
C_w	Concentration of substance dissolved in the bulk liquid
C_{s+w}	Concentration of total dissolved and sorbed substance
°C	Degrees Celsius
cc	cubic centimeters (cm^3)
CFSTR	Continuous flow stirred tank reactor
C,N,P	Carbon, nitrogen and phosphorous
CWA	Clean Water Act (US)
d	Durbin-Watson's statistic; tests correlation in residual error
DCM	Dichloromethane (methylene chloride)
DMQ	Double MilliQ® deionized
DNA	Deoxyribonucleic acid
DRC	Dose response curve
E1	Estrone
E1-3S	Estrone-3-sulphate
E1-3G	Estrone-3-glucuronide
E2	Estradiol
E2-3S	Estradiol-3-sulfate
E2-3G	Estradiol-3-glucuronide

List of Symbols and Abbreviations(cont'd)

Abbreviation	***Definition***
E2-Eq	RYA determined estradiol equivalents
E3	Estriol
E3-3S	Estriol-3-sulfate
E3-3G	Estriol-3-glucuronide
E3-16G	Estriol-16-glucuronide
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting chemical
EDTA	Ethylenediaminetetraacetic acid
EEq	Chemically determined estradiol equivalents
EE2	Ethinylestradiol
EE2-3S	Ethinylestradiol-3-sulphate
EE2-3G	Ethinylestradiol-3-glucuronide
eEDC	Estrogenic endocrine disrupting chemical (Environmental estrogens)
ELISA	Enzyme linked immuosorbent assays
ER	Oestrogen receptor
F/M	Food-to-microorganism ratio
Fr#	SPE fraction number
FECp	Final effluent composite (pre-UV)
FEC	Final effluent composite (post-UV)
g, mg, µg, ng	Gram, milligram, microgram, nanogram
gSS/m^3	Gram suspended solids per cubic meter wastewater
GC-HRMS	Gas chromatography – high resolution mass spectrometry
GC-MS	Gas chromatography – mass spectrometry (Low resolution)
h	Hours
hER	Human estrogen receptor
HLC	Henry's Law constant
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time

List of Symbols and Abbreviations(cont'd)

Abbreviation	***Definition***
IS	Internal standard
ISM	Internal standard mixture
k_{bio}	Psuedo-first order biological reaction rate constant
k_{sor}	First order sorption rate constant
K_D	Solid-liquid partitioning coefficient
K_{oc}	Organic carbon / water partitioning coefficient
K_{ow}	Octanol / water partitioning coefficient
kPA	Kilopascal
L, mL, µL	Liter, milliliter, microliter
L/gSS	Liter per gram suspended solids
L/gSS·d	Liter per gram suspended solid days
LC-MS	Liquid chromatography – mass spectrometry
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LOEL	Lowest observable effects level
m, µm, nm	Meter, micrometer, nanometre
$\sum m$	Total mass flux over seven-day period
MBR	Membrane biological reactor
MeOH	Methanol
MDL	Method detection limit
MLD	Megaliters per day
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MPW	Membrane (UF) permeate water
MWWT	Municipal wastewater treatment
m/z	Mass / charge
NPEOs	Nonylphenol polyethoxylates
n	Sample size
ND	Non-detect
na	Not available

List of Symbols and Abbreviations(cont'd)

Abbreviation	***Definition***
ng/gSS	Nanograms per grams suspended solids
NH_3-N	Ammonia nitrogen
NH_3	Ammonia
NIST	National Institute of Standards and Technology
NP	Nonylphenol
OWC	Organic wastewater contaminants
p	Minimum α / type II error to detect a significant difference
PAOs	Phosphorous accumulating organism
PCBs	Polychlorinated biphenyls
PCR	Polymerized chain reaction
PE	Primary effluent
pH	-Log $[H^+]$
PPCPs	Pharmaceuticals and personal care products
PR	Percent removal
psi	Pounds per square inch
PVC	Polyvinylchloride
Q	Wastewater flow rate
QA/QC	Quality assurance / quality control
r	Pearson's correlation coefficient
r^2	Coefficient of determination
RAW	Untreated wastewater (plant inlet)
%Red	Percent reduction
RIA	Radioimmunoassays
%RSD	Percent relative standard deviation
rtER	Rainbow trout estrogen receptor
RVS	Removal via adsorption to new sludge
RYA	Recombinant yeast screen (measures in E2-equivalents)
S	Effluent biochemical oxygen demand
S_0	Influent biochemical oxygen demand

List of Symbols and Abbreviations(cont'd)

Abbreviation	***Definition***
SD	Standard deviation
SIM	Single ion monitoring
SPE	Solid phase extraction
SRT	Solids retention time
SS	Suspended solids (i.e. TSS)
$\overline{SS}_{production}$	Mean sludge production (gSS / m^3 wastewater)
TF	Trickling filter
TF/SC	Trickling filter / solids contact
TIE	Toxicity identification and evaluation
TKN	Total Kjeldahl nitrogen
TMS	Trimethylsilyl-derivative
TMCS	Trimethylchlorosilane
TRE	Toxicity reduction evaluation
TSS	Total suspended solids
USEPA	United States Environmental Protection Agency
V	Reactor volume
VSS	Volatile suspended solids
Vtg	Vitellogenin
UV	Ultraviolet
w/w	weight / weight
w/v	weight / volume
WWTP	Wastewater treatment plant
X_t	RYA measured E2-Eq on day t
Y_{obs}	Observed biomass yield for aerobic heterotrophs
Y_{NOx}	Biomass yield for nitrifying bacteria

Chapter1 - Introduction

1.1 Background

Environmental estrogens are a subset of chemicals which make up a group of environmentally important compounds known as endocrine disrupting compounds (EDCs). EDCs have been the subject of a plethora of research studies in biology, toxicology, chemistry, environmental science and engineering published in a wide variety of journals starting in the early 1990s.

1.1.1 Why all the concern?

The term "endocrine disrupter" was coined by a group of experts organized by Theo Colborn, a WWF biologist at the Wingspread Conference Center, in Racine, Wisconsin, July 1991 in a work session on "Chemically-induced alterations in sexual development: the wildlife/human connection". At the time, most of the evidence based on wildlife studies documenting adverse impacts including thyroid dysfunction, decreased fertility, decreased hatching success, gross birth deformities, metabolic abnormalities, behavioral abnormalities, feminization and compromised immune systems in several species of birds, fish and/or mammals (Colborn and Clement 1992). Potential mechanism of action for EDCs may involve modulation of endocrine function including aspects of biosynthesis, transport or availability, and metabolism of endogenous hormones (see Figure 1-1). More importantly, the activities of hormones may be affected by EDCs directly interacting with hormone receptors and signaling processes (Lister and Van der Kraak 2001).

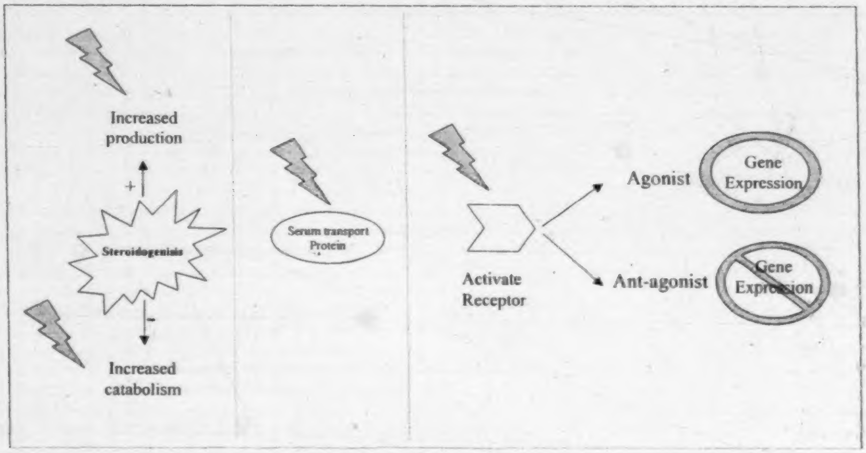


Figure 1-1. Various modes of action (thunderbolts) possible for EDCs.

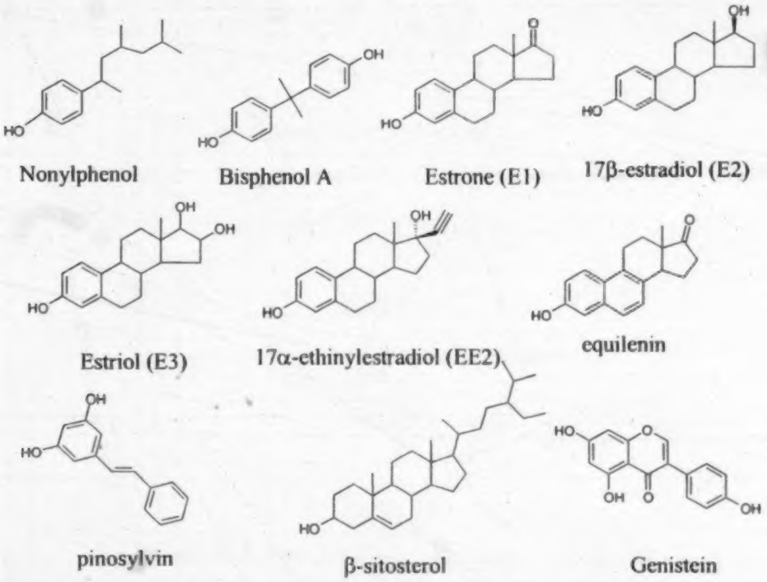
One of the largest bodies of evidence illustrating endocrine disruption in wild organisms due to an anthropogenic stressor is the scientific literature reporting reproductive effects in several fish species in association with municipal wastewater effluents and pulp/paper industry discharges (Denton et al. 1985; Jobling et al. 1998; Reviewed in Vos et al. 2000). In 2003, a team of scientists from West Virginia Division of Natural Resources and the U. S. Geological Survey found a high incidence of an intersex condition, oocytes in the testes, among smallmouth bass (*Micropterus dolomieu*) in the South Branch Potomac River and the Cacapon River of West Virginia, indicating the possible presence of EDCs (Chambers and Leiker 2006). Chowen and Nagler (2004) found that 33-57% of physiological female Chinook salmon from three naturally spawning populations in the Columbia River tested positive for a male-specific DNA marker. Due to their obligate aquatic respiration and osmoregulatory mechanisms, fish show an increased risk of exposure to EDCs and other aquatic pollutants (Damstra et al. 2002). One of the proposed mechanisms for sex reversal of these genetically male salmon involves exposure to "environmental estrogens". Domestic and industrial wastewater are well known primary point-source inputs for many contaminants of concern in aquatic environments (Liu and Lipták, 1999). The role of endogenous 17β-estradiol (E2) [1.1] (see all chemical information and structures in Appendix I) in maintaining reproductive health and regulating development, as well as its effects on growth, metabolism, and immunity in vertebrates makes potential exposure to xenoestrogenic substances of particular concern (Mommsen and Moon 2005).

In 1999 and 2000, the U.S. Geological Survey used five newly developed analytical methods to measure the concentrations of 95 organic wastewater contaminants in water samples from a network of 139 streams across 30 states. The compounds in question included antibiotics and other prescription drugs, non-prescription drugs, steroids, reproductive hormones, personal care products, products of oil use and combustion, and other extensively used chemicals (Kolpin et al. 2002). The selection of stream sampling sites was biased toward those downstream of intense urbanization and livestock production. Non-prescription drugs such as acetaminophen, caffeine, and cotinine (metabolite of nicotine) were detected in 21-74% of the streams. These non-prescription drugs were found more commonly than the groups of antibiotics, other prescription drugs (i.e. analgesics, antihypertensives, antidepressants, etc), and reproductive hormones.

The frequent occurrence of these compounds in rivers and streams, many of which are used as sources of drinking water, raises questions as to whether the compounds will remain in finished drinking water, and what the effect on human health may be. Vieno et al. 2005 reported that a drinking water treatment plant located downstream from a WWTP in Finland produced water containing part-per-trillion (ppt) levels of ibuprofen and ketoprofen in a winter sample. Based on the concentrations of pharmaceuticals reported in various drinking water studies (Stackelberg et al. 2004; Vieno et al. 2005;Chen et al. 2005), an individual would need to consume thousands of liters in a day to receive anywhere near the pharmacologically active dose of some of the compounds found most commonly in drinking water (i.e. carbamazepine, caffeine, ibuprofen). However, the human health implications of chronic exposure to low levels of some of the more potent drugs remain unknown, as does the effect of interactions at these low concentrations. These questions are particularly relevant to the practice of direct or indirect water reuse.

1.1.2 Who are the culprits?

Studies attempting to pinpoint chemical culprits responsible for the aforementioned reproductive disruption in fish associated with wastewater discharge have focused on estrogenic compounds and *in vitro* activity (e.g. Desbrow et al. 1998; Cespedes et al. 2004). Additionally, numerous studies involving biological effects monitoring in wildlife utilizing various biomarkers of exposure (Chowen and Nagler 2004; Jobling et al. 1998; hAfonso et al. 2002; McMaster et al. 2001), *in vitro* bioassays (Pelissero et al. 1993; Soto et al. 1995; Routledge and Sumpter 1996; Gaido et al. 1997; Legler et al. 1999) and chemical analyses for specific compounds (Ternes 2001; Vanderford et al. 2003; Benijts et al. 2004; Snyder et al. 1999) have been performed in attempt to bridge the gap in cause and effect for environmental estrogens. The compounds shown in Figure 1-2 represent typical environmental estrogens or estrogenic endocrine disrupting compounds (eEDCs) which have been identified as likely culprits of reproductive disruption in aquatic organisms in the vicinity of wastewater discharge zones.



Nonylphenol

Bisphenol A

Estrone (E1)

17β-estradiol (E2)

Estriol (E3)

17α-ethinylestradiol (EE2)

equilenin

pinosylvin

β-sitosterol

Genistein

Figure 1-2. Structures and common names for typical eEDCs in wastewater

The eEDCs include industrial chemicals (nonylphenol [1.2], bishpenol A [1.3]), natural and synthetic steroidal estrogens (E1 [1.4], E2 [1.1], E3 [1.5], EE2 [1.6] and equilenin [1.7]), and various naturally occurring phytoestrogens (pinosylvin [1.8], β-sitosterol [1.9], genistein [1.10]). The structures of organic chemicals capable of binding with human or fish estrogen receptors (ERs) vary widely reflecting the promiscuous nature of the ligand – receptor relationship observed with these receptors (Elsby et al. 2000). Ultimately, this mixed class of compounds along with a few of their parent compounds and metabolites have been the focus of much of the chemical and bio-analytical work performed to assess the potential exposure of aquatic organisms to environmental estrogens from municipal and industrial wastewaters. Nevertheless, efforts to discover new and, thus far, unidentified sources of estrogenicity or other hormonal effects in the environment are still currently underway.

1.2 Statement of Objectives

1. Perform a survey of estrogenicity and individual concentrations of known potent eEDCs, and if needed toxicity identification and evaluation (TIE) assessment of effluents collected from various wastewater treatment plants in Canada.

2. Refine and optimize analytical methods for the detection of priority eEDCs identified in objective 1 both in dissolved and adsorbed forms, as well as, develop a suitable method to detect conjugated eEDCs dissolved in wastewater.

3. Perform an in-depth mass balance and temporal monitoring of priority eEDCs along with net estrogenicity in select treatment plant(s) using select treatment processes (i.e. conventional activated sludge) by collecting samples isolating important unit operations and processes.

1.3 Literature Review

1.3.1 Sources of Literature

Sources consulted for the review of current and pertinent literature in the area of eEDCs in municipal wastewater include peer reviewed literature such as research articles in *Environmental Science and Technology*, *Water Environment Research*, *Water Science and Technology*, *Water Research* and *Environmental Toxicology and Chemistry*. Additionally, conference proceedings from annual Society of Environmental Toxicology and Chemistry (SETAC) meetings which were attended by the author of this thesis were included. Furthermore, general searches were performed using the combined Compendex®, Ei-Backfile, Inspec®, Inspec Archive, NTIS™ database, and Scopus® through the University of Alberta electronic library. These databases allow searching on the broadest possible range of topics within the scientific, applied science, technical and engineering disciplines and include journal articles, proceedings, and unclassified government reports from 1884 to present.

1.3.2 Chemical Methods for Determination of eEDCs in Wastewater

The chemical determination method of choice for a large number of semi-volatile organic compounds such as the eEDCs illustrated in Figure 1-2 is normally gas chromatography - mass spectrometry (GC-MS) (Petrovic et al. 2002). In most cases, derivatization of the target analytes is needed to improve peak-shape and provide adequate sensitivity by GC analysis (Kitson et al. 1996). However, the high polarity of some members of this group (e.g. genistein [1.10], estriol [1.5]), generally results in a lower thermal volatility and poor trimethylsilyl(TMS)-derivatization efficiency of these analytes and thus, poor quantification via conventional GC methods. Many liquid chromatography - mass spectrometric (LC-MS) based methods have been developed for the analysis of highly polar eEDCs and other pharmaceutical compounds in wastewater and receiving waters (Reviewed in Richardson 2004). LC-MS has been the method of choice for the ever growing list of emerging pharmaceuticals in wastewater including antibiotics, anti-inflammatory, anti-epileptic, anti-cholesterol, and anti-arthritic and oral contraceptive drugs (Note: pharmaceuticals are not covered in this review, however this emerging contaminant group does include a synthetic birth control drug, EE2 [1.6] and hormone replacement therapy drug, equilenin [1.7] which are both included in this work) which also tend to be quite polar, thermally labile and have low volatility (Vanderford et al. 2003). Tandem MS/MS techniques are also popular in the analysis of environmental estrogens and other pharmaceuticals and have the advantage of increasing the signal/noise ratio and hence lowering the limit of detection in the presence of matrix interferences (Petrovic et al. 2002). MS/MS has the added advantage of decreasing sample preparation time, as clean-up steps can be minimized, and reducing the potential for analyte interferences and false positives (Richardson 2004).

Advantages of the LC-MS methods over GC-MS include higher precision and capability of analyzing larger molecular weight and polar compounds such as conjugated steroids (Ternes 2001; Petrovic et al. 2002). Many natural and synthetic steroidal estrogens are known to be excreted by humans into the sewers as polar conjugates with glucuronic acid [1.11] and sulfate [1.12] (Ternes et al. 1999). However, GC-MS techniques have the advantage of allowing for detailed full spectral scans which can be easily matched to spectral libraries (i.e. NIST, Wiely) for the identification of unknowns. Additionally, the chromatographic resolution offered by GC is far superior to that offered by LC due to the lack of eddy diffusion (multiple paths of different lengths traveled by solute) which is a major cause of band broadening and loss of resolution in the latter technique (Harris 2007). This means that GC techniques can simultaneously analyze for more compounds than LC methods which may be advantageous if speed of analysis is important.

Since many estrogenic compounds such as EE2 [1.6] have been reported to elicit adverse biological effects at concentrations as low as 1 ng/L (Jobling et al. 2003), limit of detection for this substance is a major concern when selecting an appropriate analytical method. Table 1-1 summarizes the overall method detection limits (MDLs) for various GC-MS (and MS/MS) based methods along with LC-MS (and MS/MS) based methods for several important eEDCs.

Table 1-1. Comparisons of the MDLs between several analytical methods for the determination of eEDCs in surface water and wastewater.

Method	*Volume*	*Number of eEDCs*	*MDLs*
GC-MS (ion trap) (Ternes 2001)	0.5-1L	6	1.0-2.0 ng/L
LC-MS/MS (Vanderford et al. 2003)	1L[c]	27[a]	1.0 ng/L
LC-MS (Cespedes et al. 2004)	na	27	20-220 ng/L
LC-MS/MS (Benijts et al. 2004)	500 mL	35[b]	0.1-20 ng/L
GC-MS/MS (Belfroid et al. 1999)	1L	4	0.1-2.4 ng/L
HPLC-fluorescence (Snyder et al. 1999)	5L[c]	6	2-52 ng/L

[a]Several emerging pharmaceuticals are included; [b]Several pesticides (parabens, triazines, and carbamates) were included; [c]Surface water only, no wastewater was extracted.

The general order for the limits of quantification observed for the techniques described here applied to eEDCs in complex environmental matrices is LC-MS>GC-MS/MS>LC-MS/MS as compared by Croley et al. 2000, which is in agreement for the most part with the MDLs summarized in Table 1-1 from various literature sources.

1.3.3 Bio-analytical Methods for Determination of eEDCs and Estrogenicity in Wastewater

In addition to chemical determination, a number of bio-analytical techniques and assays are available or being developed for the analysis of eEDCs in complex environmental samples. Enzyme linked immunosorbent assays (ELISAs) (Hirobe et al. 2006; Estevez-Alberola and Marco 2004) and radioimmunoassays (RIAs) (Snyder et al. 1999) have been developed to assess eEDCs in environmental samples and overcome the problem of high matrix interference. These bio-analytical methods may appear to be more robust than chemical methods due to the higher specificity and minimal to no interference from most matrix components. However, with immunoassays, higher detection limits (~µg/L range) for most common eEDCs and cross reactivity with non-target compounds have led to their limited popularity for assessing eEDCs in complex environmental samples. They may be attractive from a high throughput, low cost analysis point of view which makes them good candidates for routine methods of analysis for eEDCs in environmental discharges if the levels of eEDCs become regulated by legislative authorities.

The eEDC analytical methods mentioned thus far provide a direct measure of specific compounds thought to be contributing to endocrine disrupting effects, however, metabolic transformation of non- or weak estrogens may lead to bio-activation or bio-inactivation of compounds not currently measured by these targeted methods. For instance, daidzein [1.13], which is a flavonoid similar in structure to genistein [1.10] is reductively metabolized to a much more potent estrogen, equol [1.14] (Safe and Gaido 1998). A plethora of *in vitro* ER binding assays, and transcriptional assays including both reporter gene assays and cell proliferation assays have been developed (Anon. 2002) to use in combination or as stand alone techniques in the assessment of eEDCs in complex environmental matrices (Gomes et al. 2003). Several recombinant strains of *Saccharomyces cerevisiae* have been developed which incorporate the human estrogen receptor (hER) gene in the main chromosome of the yeast, in addition with expression plasmids carrying the report gene *lac*-Z encoding for the enzyme β-galactosidase (Routledge and Sumpter 1996; Gaido et al. 1997; Garcia-Reyero et al. 2001). Upon activation of the hER receptor, this enzyme is secreted into the medium and may be assayed using a chromogenic substrate. Advantages of using yeast for eEDCs include ease of manipulation, rapid attainment of stable transformants, ability to process large samples and a limited metabolic capacity compared with more elaborate bioassay systems (Gaido et al. 1997).

Although yeast assays are superior from a cost and convenience view point, there are limitations to this relatively simple biological system which must be kept in mind when using it to assess potential environmental pollutants. Layton et al. 2002 show that the activity of strongly hydrophobic chemicals (i.e. hydroxylated

PCBs) may be underestimated using a recombinant yeast screen. Other *in vitro* assays based on mammalian or fish cell lines have also been used to assess eEDCs in environmental samples. The E-screen is a widespread and relatively simple bioassay for environmental estrogens based on the cell number achieved by similar inocula of MCF-7 human breast cancer cells in the presence of a test substance and a negative control (Soto et al. 1995). Legler et al. 1999 developed an ER-mediated chemical-activated luciferase reporter gene-expression (ER-CALUX®) assay based on human T47D breast cancer cell line which is commercially available through BioDetection Systems (Amsterdam, The Netherlands). Petit et al. 1997 developed a bioassay based on male trout hepatocyte cultures where the naturally occurring vitellogenin (Vtg) gene expression is dependent on estradiol [1.1] exposure. Vitellogenin is the precursor to egg yolk proteins, produced in oviparous vertebrates in response to estrogens (Sumpter and Jobling 1995). In Petit et al. 1997, the authors show that only 50% of the 49 EDCs (including fungicides, herbicides, insecticides, plasticizers, detergents, PCBs, and phytoestrogens) tested exhibited estrogenic activity in both trout hepatocyte and a recombinant yeast system (also developed by the author) which expressed the rainbow trout estrogen receptor (rtER). Thus, differences in cell permeability, metabolism, toxic response, and transcriptional mechanisms are likely significant factors contributing to discrepancies in estrogenic response between assays.

Not surprisingly, the most toxicologically accurate bioassays and those normally used for regulatory purposes are based on endpoints observed in whole organisms exposed to test substances or mixtures. The rodent uterotrophic assay is the most commonly used assays for estrogenic activity (Odum et al. 1997), however a variety of fish species including rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), fathead minnow (*Pimaphales promelas*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) have also been extensively used in exposure experiments for environmental estrogen effects (Ankley et al. 1998; Nilsen et al. 2004). However, this gain in biological accuracy is normally accompanied by a loss in endpoint precision due to the inherent variability in dealing with individual organisms compared to tissue / cell cultures. Additionally, whole organism bioassay are not practical when dealing with limited amounts of substances (i.e. linking chemical data with biological activity normally requires fractionation of test substances with a reasonable degree of precision), require specialized laboratories equipped to raise animals, and are quite time consuming depending on the life-cycles of the test organisms.

1.3.4 Concentrations of eEDCs in Wastewater

Examples of concentrations of pertinent eEDCs (Figure 1-2) found in wastewater as determined using chemical methods are summarized in Table 1-2. One of the first observations that become apparent when comparing the MDLs in Table 1-1 with the results in Table 1-2, is that many of the synthetic and natural estrogens occur at concentrations similar in magnitude to the method detection limits for these analytes in wastewater. As a result, there is less reliability in the data presented for these compounds. Also, these values represent concentrations in the effluent prior to dilution in the receiving waters. Typical final effluent flow rates for municipal wastewater treatment systems may be on the order of hundreds of million L/day which from Table 1-2, one can see could lead to gram quantities of eEDC inputs per day to the receiving environment.

There are limited data available for the concentrations of eEDCs in natural waters impacted by industrial / municipal effluents due to detection level issues and the difficulty and expense involved in performing an accurate plume model with concentration profiles for these substances. However, Kolpin et al. 2002 performed an extensive survey (139 streams across 30 US states) of 95 organic wastewater contaminants (OWCs) including most of the eEDCs discussed here. Nonylphenol [1.2] was one of the most frequently detected eEDCs with a range of 0.8 - 40 μg/L, followed by bisphenol A [1.3] at 0.1-12 μg/L. Several of the synthetic and biogenic estrogens discussed in this review were detected in 5-20% of the samples (n = 70-85), one of the most common being estriol (E3) [1.5].

Ternes et al. 1999 found that out of 6 eEDCs measured in 15 sewage impacted German rivers and streams only estrone (E1) [1.4], was detected (1.6 ng/L). Although phytoestrogens were not specifically surveyed by Kolpin et al. 2002, the animal sterols coprostanol [1.15] and cholesterol [1.16] were detected most frequently out of all 95 OWCs surveyed at 0.01-150 μg/L (detection frequency = 85.7%), and 1-10 μg/L (detection frequency = 84.3%), respectively.

Table 1-2. Typical concentrations of eEDCs in wastewater effluent in North America and Europe.

Compound(s)	*Matrix*	*Concentrationl*	*Reference*
Plant Sterols	Pulp/paper mill effluents	77.4-555 μg/L	Cook et al. 1997
Genistein [1.10]]	Kraft mill effluent	10.5μg/L	Kiparissis et al. 2001
Bisphenol A [1.3]	Municipal wastewater	181 ng/L	Current Author's work
Equilenin [1.7]	Municipal wastewater	7.2 ng/L	Current Author's work
Pinosylvin [1.8]	Kraft mill effluent	6.4 ng/L	Current Author's work
E1 [1.4]	Municipal wastewater	5 ng/L	Reviewed in Johnson and Sumpter 2001
E2 [1.1]		1.5 ng/L	
E3 [1.5]		20 ng/L	
EE2 [1.6]		0.5 ng/L	
Nonylphenol [1.2]		2 μg/L	

Figure 1-3, represents the estrogenic activity as detected using the recombinant yeast screen discussed previously in various wastewaters from North America and Europe.

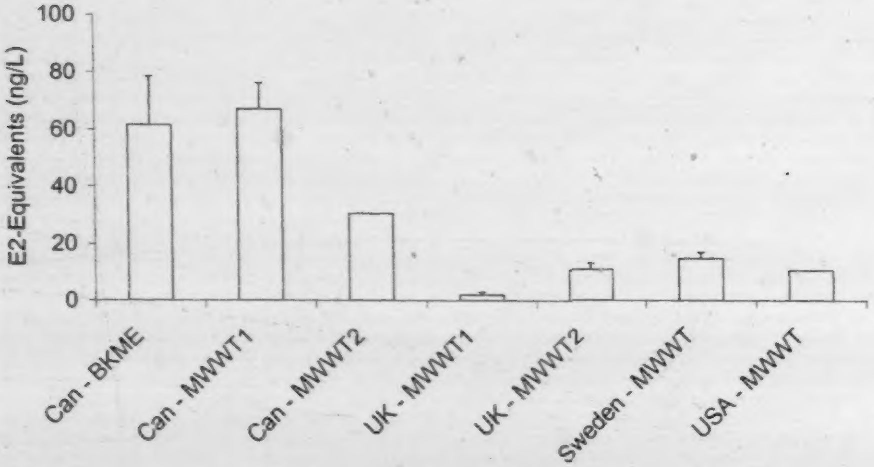


Figure 1-3. hER levels determined using recombinant yeast screens in bleached kraft mill effluent (BKME) and various municipal wastewater treatment (MWWT) effluents (Can = Current author's work; UK = Kirk et al. 2002; Sweden = Svenson et al. 2003; USA = Holbrook et al. 2002). Error bars are in units of ±1 standard deviations as measured from replicate analyses.

Table 1-3. Relative *in vitro* potencies (Gomes et al. 2003) and contributions to the estrogenicity in typical wastewater effluents for seven select eEDCs.

Compound	*Yeast Assay*	*E-screen*	*Typical Levels*[a]	*EEq*
E2 [1.1]	1	1	1.5 ng/L	1.5 ng/L
EE2 [1.6]	0.5	0.91	0.5 ng/L	0.4 ng/L
E1 [1.4]	0.3	0.096	5 ng/L	0.2 ng/L
Equilenin [1.7]	N/A	0.096[b]	7.2 ng/L	0.7 ng/L
E3 [1.5]	0.002	0.071	20 ng/L	0.7 ng/L
Nonlyphenol [1.2]	0.001	0.0001	2000 ng/L	1.1 ng/L
Bishpenol A [1.3]	0.00006	0.00004	181 ng/L	0.01 ng/L
Totals	-	-	**2215 ng/L**	**4.6 ng/L**

[a] From Table 1-2. [b] Equilenin [1.7] was found by Soto et al. 1995 (developers of the E-screen) to produce a relative proliferation rate similar to that of E1 [1.4], thus we have assigned the same potency as E1 [1.4] to this compound.

Factoring in the relative potencies (EC_{50} of β-estradiol / EC_{50} test substance) for select eEDCs determined using pure substances with the recombinant yeast assay and E-screen (the average of both assays was used), we can determine an approximated overall estrogenic equivalence (EEq) for a typical wastewater profile (see Table 1-3).

The major phytoestrogens included in Figure 1-2, β-sitosterol [1.9], genistein [1.10] and pinosylvin [1.8] are all classed as weak ER agonists (Anon. 2002; Mellanen et al. 1996). However, the data for these compounds are limited and thus, were not included in Table 1-3. Nevertheless, the overall estrogenic effects of these compounds and the possibility of them to act as precursors of strong estrogenic substances must not be overlooked due to the relatively high concentrations of these compounds discharged into receiving environments. In Table 1-3, 4.6 ng/L EEq, provides an estrogenic contribution for all seven compounds, which are some of the most commonly encountered and active eEDCs in the environment. However, when comparing this value to Figure 1-3, in most cases (i.e. exception: UK-MWWT1 - final effluents E2-equivalents = 2 ng/L) there is still a large percentage, 57-93% of unexplained estrogenic activity in wastewater effluents based on these generalized results. Murk et al. 2002 reported discrepancies as high as 80% in wastewater effluents using an ER-CALUX® bioassay versus chemically determined E2-equivalents. Potential explanations for the large discrepancy between chemical estimation of estrogenicity (assuming E2-like response) and observed net estrogenic activity *in vitro* include unidentified chemical species which need to be incorporated into the targeted chemical analysis, several weak or non-estrogens may be bio-activated *in vitro* (Petit et al. 1997), and finally, the presence of any anti-estrogens in the mixture will lower the net estrogenic response for that mixture. In addition, the EEQ approach assumes that the dose-response curves (DRC) for the test chemicals have the same slope and maximum response of the reference compound (i.e. E2) and thus, any deviations in the actual DRC may cause a loss of proportionality for the measured compounds (see Appendix XVII for a detailed explanation of this phenomenon).

In order to elucidate structures of new and unknown estrogens which may be contributing to the total estrogenicity as determined in *in vitro* assays but not accounted for by existing analytical methods, many researchers have used the framework of the toxicity identification and evaluation (TIE) approach (Gomes et al. 2003). TIE involves a multi-tiered procedure starting with the most crude sample manipulations (i.e. SPEs, pH, EDTA, etc) and moving to finer manipulations (HPLC fractionation, GC-MS identification) all guided by biologically relevant endpoints (i.e. recombinant yeast screen or E-screen). The last stage of TIE involves adding the identified culprit(s) back into the original matrix to confirm an increase in the biological endpoint. TIE procedures are an integral part of USEPA's toxicity reduction evaluation (TRE) process which is used to identify and reduce or eliminate sources of effluent toxicity regulated by the Clean Water Act (CWA) (Anon. 1999). This type of procedure was used to identify many of the known eEDCs mentioned in this proposal (Desbrow et al. 1998). Recently, Burnison et al. 2003 used a modified TIE approach to successfully identify E2 [1.1], E1 [1.4] and the phytoestrogen metabolite, equol [1.14] as major agents responsible for the overall estrogenicity of hog manure and agricultural runoff.

1.3.5 Reduction Efficiencies and Mass Balance of eEDCs in WWTPs

The issue of eEDCs in wastewater has been recognized by scientists around the world and attention has been given to the wastewater treatment process as a solution to mitigate current and future environmental damages from eEDCs. In Canada, the city of Toronto has already implemented the *Water and Wastewater Services Division Sewer Use By-law* (By-law No. 855-2002) with Pollution Prevention Planning requirements regulating the levels of specific eEDCs, nonylphenol polyethoxylates (NPEOs) [1.17] and nonylphenols (NPs) [1.2], allowed in the sewer system. Table 1-4 shows the typical reduction efficiencies for select eEDCs in wastewater in North America and Europe.

Table 1-4. Typical reduction efficiencies for eEDCs in wastewater treatment.

Compound(s)	*Treatment*	*Reduction (%)*	*Reference*
E1 [1.4]	AST	59-90%	Johnson et al. 2005
E2 [1.1]	AST	>85%	Johnson and Sumpter 2001
E3 [1.5]	AST	>85%	Johnson and Sumpter 2001
EE2 [1.6]	AST	>85%	Johnson and Sumpter 2001
Nonylphenol (NP) [1.2]	AST	37-77%	Birkett and Lester 2003
Plant Sterols	AST&ASB (pulpmills)	64-79%	Cook et al. 1997
Bisphenol A [1.3]	AST	>90%	Drewes et al. 2005
Estrogenicity	AST&TF	>70%	Kirk et al. 2002

AST: Activated sludge treatment; ASB: Aeration stabilization basin; TF: Trickling filtration.

The order for general reduction efficiencies of target eEDCs shown in Table 1-4, via different types of treatment processes is: membrane biological reactors (MBRs) > AST with nutrient removal > AST without nutrient removal > trickling filtration (Drewes et al. 2005; Shi et al. 2004; Kirk et al. 2002; Joss et al. 2004; Andersen et al. 2003).

A national survey of 28 WWTPs was conducted by Environment Canada's Wastewater Technology Center and National Water Research Institute from 1997-2000 to investigate the occurrence, fate, and release of EDCs in Canadian WWTPs. A strong correlation between operational process parameters including hydraulic retention time (HRT), sludge age (SRT) and food to micro-organism (F/M) ratio, and EDC reduction efficiency was found (Anon. 2000). The conclusions of this study recommend that a better understanding of the EDCs removal mechanisms is required to optimize and predict the removal efficiency of EDCs in WWTPs. Recently, a few studies have been performed to attempt to provide a more holistic model on optimization for EDC reduction with a focus mainly on activated sludge secondary treatment facilities. Drewes et al. 2005 reported a significant correlation ($r^2 = 0.76$) between influent BOD_5 loading and influent E2-equivalents loading in seven full-scale water reclamation facilities. Other researchers have shown that there is a positive correlation between HRT and SRT and removal of eEDCs in full-scale facilities (Holbrook et al. 2002; Johnson et al. 2005). The greater age of sludge associated with higher recycling rates increases the concentration of slower growing, perhaps specialist organisms which degrade estrogens (Joss et al. 2004). Furthermore, an increase in HRT increases the contact time between the dissolved estrogens and mixed liquor suspended solids (MLSS) and thus, increases the time available for adsorption and biodegradation to occur.

In general, there are three removal pathways for organic pollutants during secondary biological treatment: 1. adsorption onto the microbial flocs and removal in the waste sludge; 2. biological or chemical degradation; 3. transformation / volatilization during aeration (Birkett and Lester 2003). Thus, the behavior of EDCs in WWTPs is dependent on physicochemical properties of each chemical including aqueous solubility (mg/L), organic carbon / water partitioning coefficients (K_{oc}), octanol-water partitioning coefficients (K_{ow}), Henry's law constant (HLC), and molecular structure properties influencing biodegradation / bio-transformation (Birkett and Lester 2003). Recently, sorption and biological degradation models shown in Figure 1-4 for steroidal estrogens (i.e. E1[1.4], E2[1.1] and EE2[1.6]) in WWTPs have been proposed by Joss et al. 2004.

Where k_{bio} and k_{sor} are pseudo-first order reaction rate constants for biodegradation and sorption of dissolved estrogens; SS = suspended solids (i.e. MLSS or MLVSS); C_w = bulk soluble concentration; C_s = mass sorbed per unit reactor volume; K_D is the sorption coefficient = C_s / [SS x C_w] in equilibrium and is proportional to K_{ow}. 17β-estradiol-glucuronide [1.18], estrone-glucuronide [1.19], and ethinylestradiol-glucuronide [1.20] are typical conjugates of E2 [1.1], E1 [1.4] and EE2 [1.6], respectively, known to be excreted by females into the sewers.

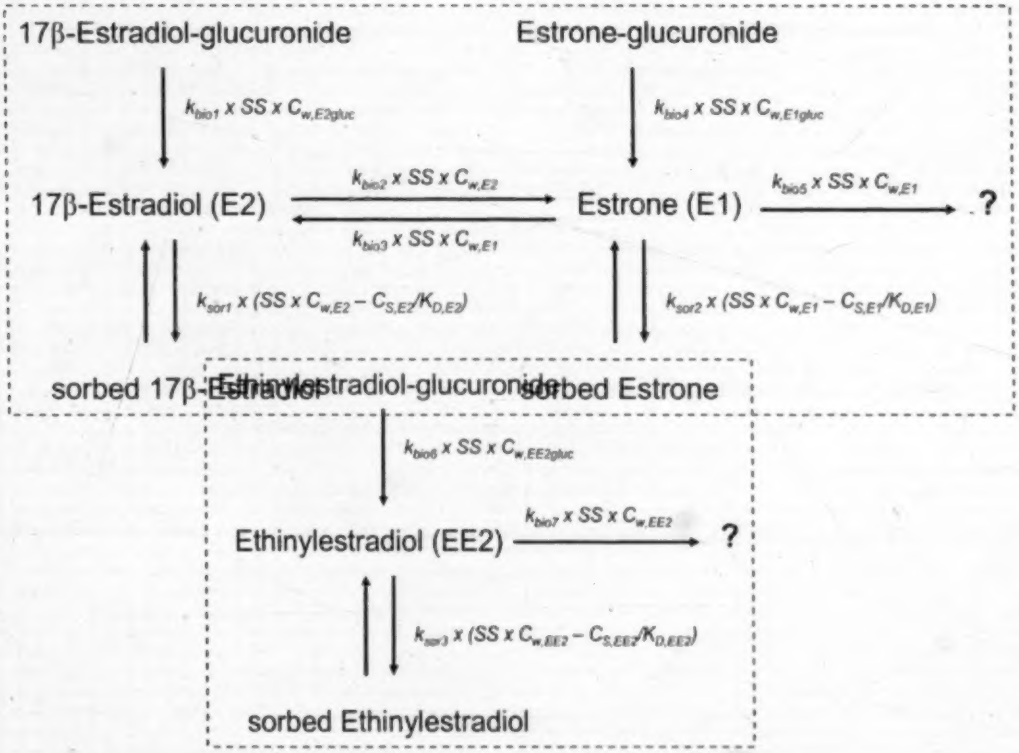

17β-Estradiol-glucuronide
Estrone-glucuronide
k_{bio1} x SS x $C_{w,E2gluc}$
k_{bio4} x SS x $C_{w,E1gluc}$
17β-Estradiol (E2)
k_{bio2} x SS x $C_{w,E2}$
k_{bio3} x SS x $C_{w,E1}$
Estrone (E1)
k_{bio5} x SS x $C_{w,E1}$
?
k_{sor1} x (SS x $C_{w,E2}$ – $C_{S,E2}/K_{D,E2}$)
k_{sor2} x (SS x $C_{w,E1}$ – $C_{S,E1}/K_{D,E1}$)
sorbed 17β-Estradiol
sorbed Estrone
Ethinylestradiol-glucuronide
k_{bio6} x SS x $C_{w,EE2gluc}$
Ethinylestradiol (EE2)
k_{bio7} x SS x $C_{w,EE2}$
?
k_{sor3} x (SS x $C_{w,EE2}$ – $C_{S,EE2}/K_{D,EE2}$)
sorbed Ethinylestradiol

Figure 1-4. Sorption / biological degradation models for steroidal estrogens in WWTPs (adapted from Joss et al. 2004).

Thus, according to the model presented in Figure 1-4, the reduction efficiencies for steroidal estrogens in a secondary treatment process are governed by pseudo-first order biodegradation / bioconversion reactions dependent on dissolved free and conjugated estrogen concentrations, and suspended solids concentrations. In addition, sorption kinetics may play an important role if adsorption of a particular chemical to the floc proceeds slowly compared with the other reactions already discussed. Thus, a mass balance assessment based on free estrogen '*x*' in a continuous flow stirred tank reactor (CFSTR) would be as follows (Tchobanoglous et al. 2003; Joss et al. 2004):

$$\begin{aligned}&\frac{dC}{dt}\cdot V = C_{s+w,x(\mathrm{inf})}\cdot Q - C_{s+w,x(eff)}\cdot Q + [k_{bio1}\cdot SS\cdot C_{w,x-gluc}]\cdot V - \\ &[k_{sor}\cdot(SS\cdot C_{w,x} - C_{s,x}/K_{D,x})]\cdot V - [k_{bio2}\cdot SS\cdot C_{W,x}]\cdot V\end{aligned} \tag{1-1}$$

Where [dC/dt]·V represents any net change in total estrogen mass (i.e. unit mass / time) in the reactor; k_{bio1} is the pseudo-first order rate constant for the cleavage reaction of *x-gluc* (i.e. conjugated estrogen) and k_{bio2} is the pseudo-first order rate constant for the biodegradation of *X*; Q is the flow rate through the reactor; V is the volume of the reactor. It is assumed that the SS in the reactor effluent are negligible. In an ideal system at equilibrium the [dC/dt]·V and $C_{\mathrm{s+w,x(eff)}}$·Q terms would be zero and the sorption and degradation terms (i.e. the two right hand side terms on the bottom line) would equal the cleavage reaction and influent terms (i.e. the two right hand side terms on the upper line which remain). This would represent a 100% removal for a particular conjugated estrogen assuming there were no other removal or formation mechanisms which (1-1) does not account for.

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Chapter2-An assessment of estrogenic organic contaminants in Canadian wastewaters

This publication was accepted for publication in *Science of the Total Environment* (Fernandez et al. 2007b) on November 15th, 2006. This work represents an extensive investigation of 30 primarily estrogenic organic wastewater contaminants and parallel *in vitro* estrogenicity for wastewater from four municipal and one industrial wastewater treatment plants in western Canada. Details of the developed GC-HRMS based method (Ikonomou et al. 2007) that allowed the simultaneous detection of 30 eEDCs are referred to in the appendices. Additionally, this work reports on a detailed chemical fractionation procedure guided by the *in vitro* estrogenic screen for the identification of additional estrogenic culprits in wastewater.

2.1 Introduction

Environmental estrogens are a subset of chemicals which make up a group of environmentally important compounds known as endocrine disrupting compounds (EDCs). Potential mechanism of action for estrogenic contaminants may involve modulation of endocrine function including aspects of biosynthesis, transport or availability, and metabolism of endogenous hormones. Additionally, the activities of hormones may be affected by estrogenic contaminants interacting with hormone receptors and signaling processes (Lister and Van der Kraak 2001). One of the largest bodies of evidence illustrating endocrine disruption in wild organisms due to an anthropogenic stressor is the scientific literature reporting reproductive effects in several fish species in association with municipal wastewater effluents and pulp/paper industry discharges (Vos et al. 2000; Denton et al. 1985; Jobling et al. 1998). In North America, Nagler and colleagues (Nagler et al. 2001; Chowen and Nagler 2004) found that a high proportion of physiologically female Chinook salmon from three naturally spawning populations in the Columbia River tested positive for a male-specific DNA marker. From these results, the authors hypothesized that endocrine disruption may be playing a role in the development of the physiological sex of these fish. Such apparent sex reversal effects have not been observed in British Columbia populations (Devlin et al. 2005), but laboratory experiments have provided some evidence for sex reversal in this species arising from exposure to municipal and kraft pulp mill effluents (Afonso et al. 2002). Thus, a possible mechanism for sex reversal of genetically male salmon involves exposure to environmental estrogens originating primarily from wastewater discharged into their habitat. The human health implications of the occurrence and levels of estrogenic contaminants among other organic wastewater contaminants (OWCs) in wastewater effluents may also be important in some locations where hydrologic route between waste and drinking water is relatively short. Ubiquitous OWCs including non-ionic surfactant metabolite nonylphenol (NP) [1.2] have recently been reported in surface and drinking water at low to mid ng/L ranges (Chen et al. 2005).

In order to strengthen the relationship between potential chemical culprits and potential biological effects many researchers have used the framework of the toxicity identification and evaluation (TIE) approach (Gomes et al. 2003). TIE involves a multi-tiered procedure starting with the most crude sample manipulations (i.e. SPEs, pH, EDTA, etc) and moving to finer manipulations (HPLC fractionation, GC-MS identification) all guided by biologically relevant endpoints (i.e. recombinant yeast screen or E-screen). TIE procedures are an integral part of USEPA's toxicity reduction evaluation (TRE) process which is used to identify and reduce or eliminate sources of effluent toxicity regulated by the Clean Water Act (CWA) (Anon. 1999). This type of procedure was used to identify many of the known estrogenic contaminants targeted in this work (Desbrow et al. 1998). Recently, Burnison et al. 2003 used a modified TIE approach to successfully identify E2 [1.1], E1 [1.4] and the phytoestrogen metabolite, equol [1.14] as major agents responsible for the overall estrogenicity of hog manure and agricultural runoff. This type of technique may be important for identifying culprit compounds in wastewater to target for fate and reduction studies.

The issue of estrogenic contaminants in wastewater has been recognized by scientists around the world, and the ability of wastewater treatment processes to mitigate current and future environmental risks from these compounds is being investigated (Drewes et al. 2005; Shi et al. 2004; Kirk et al. 2002; Joss et al. 2004; Andersen et al. 2003). The objectives of the present study were to survey the concentrations, in western Canada wastewater influent and effluent samples, of typical estrogenic contaminants and other OWCs, which have been identified as likely agents of reproductive disruption in aquatic organisms in the vicinity of wastewater discharge zones. The compounds under study include industrial chemicals (e.g. nonylphenol (NP) [1.2] and phthalate esters), natural and synthetic steroidal estrogens (e.g. estrone (E1) [1.4], estradiol (E2) [1.1], estriol (E3) [1.5], 17α-ethynylestradiol (EE2) [1.6] and equilenin [1.7]) and progesterones (19-norethindrone [2.1] and (-)-norgestrel [2.2]), and various naturally occurring phytoestrogens (e.g. pinosylvin [1.8], β-sitosterol [1.9]). Sample analysis included both the determination of chemical concentrations and estrogenic activity. The concentrations of these compounds were assessed using an ultra-trace analytical method based on gas chromatography - high resolution mass spectrometry (GC-HRMS). These results were complemented by an *in vitro* assay (i.e. recombinant yeast screen) to assess the estrogenic activity in the wastewater samples. Additionally, a modified TIE protocol was applied to key municipal effluents to further investigate the relationship between the chemical and *in vitro* data. A representative cross-section of wastewaters that are commonly discharged to coastal or inland waters of western Canada was selected for sampling in this study. The impacted waters include the Fraser and North Saskatchewan rivers, both of which are major fish bearing rivers in western Canada.

2.2 Experimental methods

The details of experimental procedures appear below in sections 2.2.1-2.2.5. A general schematic of these procedures is shown in Figure 2-1.

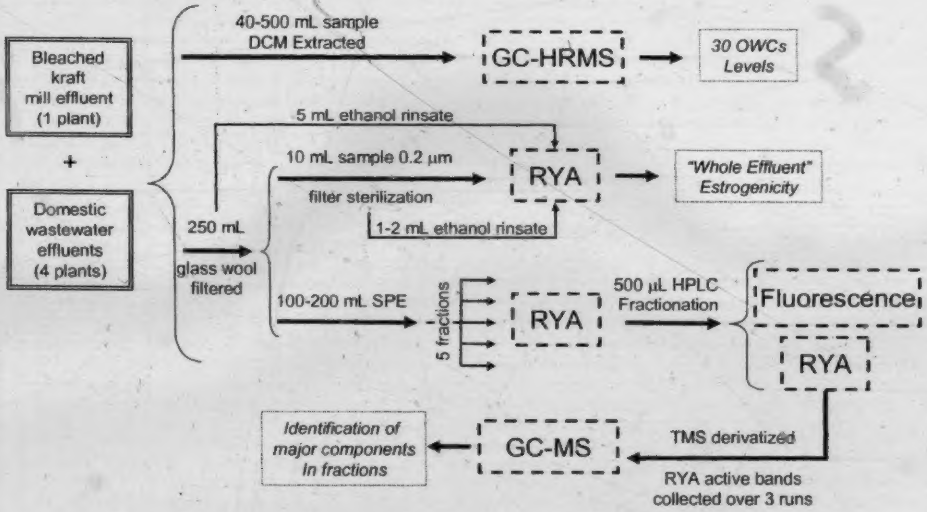

Bleached kraft mill effluent (1 plant)
+
Domestic wastewater effluents (4 plants)
40-500 mL sample DCM Extracted
GC-HRMS
30 OWCs Levels
5 mL ethanol rinsate
10 mL sample 0.2 µm filter sterilization
RYA
"Whole Effluent" Estrogenicity
1-2 mL ethanol rinsate
250 mL
glass wool filtered
100-200 mL SPE
5 fractions
RYA
500 µL HPLC Fractionation
Fluorescence
RYA
TMS derivatized
RYA active bands collected over 3 runs
GC-MS
Identification of major components in fractions

Figure 2-1. General schematic of the experimental procedures performed.

2.2.1 Sampling

Bleached kraft mill effluent (BKME) was obtained from a Canadian Northern Softwoods Kraft mill (site A; See Table 2-1) producing 257 air dried tonnes of kraft pulp per year and served by an activated sludge treatment plant.

Table 2-1. Characteristics of the Treatment Plants Surveyed.

Site	Major Source	Treatment	Average Flow (MLD)	HRT	$BOD_{5\ final}$ (mg/L)	TSS_{final} (mg/L)
A	Kraft Mill	AST	70	8hrs	10	20
B	Domestic	TF/SC	455	0.3-2hrs	12	12
C	Domestic	AST1	76	13hrs	4*	5
D	Domestic	AST2	257	8hr	6	10
E	Domestic	Lagoon	10.6	~17days	~20	~20

HRT = hydraulic retention time (biological treatment process only); MLD = mega-liters per day (average annual); BOD_5 = five-day biochemical oxygen demand (typical); TSS = total suspended solids (typical). *Carbonaceous BOD only.

The trickling filter / solid contact (TF/SC) biological secondary treatment plant (site B) with chlorine disinfection serves a suburban population of 740,000. Two activated sludge treatment plants with biological nutrient removal (AST w/BNR) treating municipal sewage serving populations of 195,000 (site C) and 720,000 (site D) were also included in the survey. The aerated lagoon (AL) (site E) system consists of 3 lagoons in series with decreasing dissolved oxygen levels and no chemical treatment. This system serves a rural population of around 20,000.

Grab samples were taken in the morning using pre-cleaned 1 or 4L glass amber bottles at sampling points established by plant personnel as indicated in Table 2-2. Flow proportional 24-hour composite samples were available for site D only. Due to the labile nature of many known environmental estrogens, immediate sample processing and preservation of stored materials was of high importance. In an attempt to minimize the impact of on-going biological and physical (i.e. UV) degradation in the samples extractions for chemical and *in vitro* analysis were performed within 48 hours of sample collection.

Table 2-2. Sampling points and time frame details.

Sample Site	Class	Prior to Point	Dates (n)
Site A - Kraft Mill	Effluent	Final outfall	30/12/02, 6/01/03, 13/01/03, 20/01/03, 27/01/03, 3/02/03 (6)
Site B - Trickling	Influent	Bioreactor	16/12//02, 23/12/02, 30/12/02, 6/01/03, 13/01/03, 20/01/03, 27/01/03, 3/02/03 (8)
Site B - Trickling	Effluent	Final outfall	16/12//02, 23/12/02, 30/12/02, 6/01/03, 13/01/03, 20/01/03, 27/01/03, 3/02/03 (8); 26/03/03 (4); 2/04/03 (4); 9/04/03 (4); 16/04/03 (4)
Site C - AST1	Influent	1°Sedimentation	26/04/05 (1)
Site C - AST1	Effluent	Final outfall	26/04/05 (1)
Site D - AST2	Influent	1°Sedimentation	26/04/05 (1)
Site D - AST2	Effluent	Final outfall	26/04/05 (1)
Site E - Lagoon	Influent	Raw	5/03/04 (1)
Site E - Lagoon	Effluent	Final	22/03/04, 25/03/04, 5/03/04 (3)

2.2.2 Targeted Chemical Analysis

The method used to determine the levels of OWCs in wastewater samples (Table 2-3 for a list of all targeted compounds) was previously developed and recently accepted for publication (Ikonomou et al. 2007). In brief, 40-500 mL of sample was spiked with five labeled / non-naturally occurring internal standards (Appendix II, Table A2-1) and subsequently extracted with approximately 3 volumes of 10-50 mL dicloromethane (DCM). Extracts were reduced under a gentle stream of nitrogen, dried over sparing amounts of sodium sulfate and cleaned up using 5% (w/w) deactivated florisil. Extracts were derivatized using 50 µL of anhydrous pyridine and 50 µL of freshly prepared bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) for 3 hours at 90°C. One microliter of each sample was injected (splitless mode) onto the GC-HRMS which was comprised of a Hewlett Packard 5890 Series II Gas Ghromatograph coupled to a VG AutoSpec magnetic sector mass spectrometer (Micromass UK Ltd., Manchester, UK). Further details of the instrumental method including chromatograms and ions monitored can be found in Figure A2-1 and under the "Instrumental analysis" heading both located in Appendix II. Quality control samples including procedural blanks, spikes and duplicates were run with each batch of 10 samples. Results are reported as recovery corrected and blank-subtracted values. Internal standard recoveries were generally >70% and never outside of 40 to 120% as a quality control criterion. Further details of the QA/QC can be found under the "QA/QC" heading in Appendix II.

A wide range of potential estrogenic culprits were selected for this work based on the literature review presented in Chapter 1. Also, certain compounds listed in Table 2-3 were compounds related to already classified eEDCs with a diverse range of sources.

Table 2-3 - All targeted compounds and their significance

Compound Name	*Description*
17α- and 17β-Estradiol [1.1]	Endogenous female estrogen
Estrone [1.4]]	Endogenous female estrogen
Estriol [1.5]	Endogenous female estrogen
17α-Ethynylestradiol [1.6]	Synthetic ovulation inhibitor (birth control pill)
Mestranol [2.3]	Synthetic ovulation inhibitor (birth control pill)
19-Norethindrone [2.1]	Synthetic ovulation inhibitor (birth control pill)
Norgestrel [2.2]	Synthetic ovulation inhibitor (birth control pill)
Equilenin [1.7]	Hormone replacement therapy drug
Equilin [2.4]	Hormone replacement therapy drug
α-Zearalanol [2.5]	Veterinary drug - growth promoter
17β-Estradiol-3-benzoate [2.6]	Veterinary drug - growth promoter
Testosterone [2.7]	Endogenous male androgen
β-Sitosterol [1.9]	Major plant derived sterol
Campesterol [2.8]	Major plant derived sterol
Stigmasterol [2.9]	Major plant derived sterol
Stigmastanol [2.10]]	Major plant derived sterol
Pinosylvin [1.8]	Stilbene found in *Pinus* species
Cholesterol [1.16]	Animal derived sterol
Coprostanol [1.15]]	Cholesterol derivative
Desmosterol [2.11]	Cholesterol derivative
Ergosterol [2.12]	Main sterol produced by fungi
6- ketocholestanol [2.13]	Cholesterol oxidation products
7-ketocholesterol [2.14]	Cholesterol oxidation products
Coprostan-3-one [2.15]	Fecal neutral sterol
Fucosterol [2.16]	Sterol found in seaweed
Totarol [2.17]	Antibacterial diterpenoid
4-Nonylphenol [1.2]	Potent xenoestrogen from non-ionic surfactants
Bis(2-ehtylhexyl) phthalate (DEHP) [2.18]	Ubiquitous plasticizer / phthalate ester
Bisphenol A [1.3]	Plasticizer primarily from PVC plastics

2.2.3 Recombinant Yeast Assay (RYA)

The recombinant yeast strain used in this work was obtained from Prof. J.P. Sumpter from Brunel University, Middlesex, UK. This yeast was previously modified to contain the DNA sequence of the human estrogen receptor (αhER) on the main chromosome, as well as, an expression plasmid carrying the reporter gene *Lac-Z* which encodes for the enzyme β-galactosidase. The assay was carried out as specified in Routledge and Sumpter 1996. For calculation of β-galactosidase activity, the ratio of absorbance at 540 nm to that at 650 nm (i.e.

optical density) was subtracted from the same ratio obtained from an appropriate blank well. Dose-response curves (DRCs) were plotted as the blank-subtracted β-galactosidase activity for twelve estrogen (E2) [1.1] standards run in triplicate versus the log of the concentration in grams per liter. The curves were fit using a sigmoidal dose-response curve (variable slope), Marquardt-Levenberg algorithm in SigmaPlot version 8.0 (SPSS, Chicago, IL, USA). Coefficients of determination (r^2) of 0.998 or better were observed for the dose-response curves. See Appendix III for more RYA methodology details, all modifications from the cited protocol and an example of a DRC obtained in this work.

Wastewater samples of 250 mL were glass wool filtered; 10 mL aliquots of the filtrate were filter sterilized using 0.2 μm Puradisc® cartridge filters (Whatman, Middlesex, UK) and stored in sterile glass vials for RYA analysis. The glass wool plugs were rinsed with 5 mL of deionized water followed by 5 mL of anhydrous ethanol, the latter of which was collected for RYA. Additionally, the 0.2 μm filters that were used to sterilize aqueous fractions were rinsed with a few milliliters of ethanol to recover any estrogenic substances lost in filter sterilization. Any activity in the ethanol rinsates was quantitatively added to the whole effluent estrogenicity. All values were blank-subtracted using blanks of double deionized water treated identically as the samples.

2.2.4 Modified Toxicity Identification and Evaluation (TIE) approach

A 100-200 mL aliquot of each wastewater sample was extracted onto a 500 mg Oasis HLB 6 mL cartridge column (Waters Corp., Milford, MA, USA) after glass wool filtration. Vacuum (<85 kPa) was used to drive the sample through the column and air-dry the column after extraction. Aliquots of the SPE eluant were collected to test for "break-through" of any estrogenic components throughout the extraction. Columns were stored at -20°C prior to elution which was performed using 5 mL of 25% methanol (MeOH) in double Milli-Q® deionized (DMQ) water, followed by 50% MeOH, 100% MeOH, diethyl ether and cyclohexane for a total of five fractions. For BKME samples the elution solvent sequence was slightly modified to address differences in polarity of this matrix and consisted of: 25% MeOH, 50% MeOH, 75:25 MeOH / ethyl acetate, methyl tert-butyl ether (MTBE) and cyclohexane. Diethyl ether, MTBE and hexane fractions were taken to dryness under N_2 (<30°C) and re-suspended in MeOH prior to estrogen screen analysis. The SPE fractionation procedure was validated for the recovery of potential estrogenic compounds using a mixture of labeled surrogate standards spiked into wastewater samples (see Section 2.3.2).

Using a syringe, 500 μL of the 100% methanol fractions (i.e. the most estrogenic SPE fractions as determined by RYA) from each site were injected via a Waters U6K injector on to a Phenomenex Luna® 5μm Silica(2) 250 x 4.60 mm semi-preparative HPLC column. A flow rate of 0.8 mL/min was used with a linear gradient starting with a mobile phase composition of 75% 3:2 DMQ water:acetonitrile and 25% 3:1 methanol:iso-propanol and ending after a 15 minute run with a mobile phase composition of 3:1 methanol:iso-propanol. A HP 1046A fluorescence detector was used with an excitation wavelength (λ_{ex}) of 225 nm and emission wavelength (λ_{em}) of 295 nm to monitor the eluant for distinct bands of the further fractionated wastewater extract. Single drops of the HPLC eluant during an injection where collected into 96-welled plates every 45 seconds for RYA analysis for the presence of estrogenic substances. A standard mixture was made up of 7 mg/L cholesterol [1.16] (i.e. a fluorescently detectable surrogate for sterols) and 150 mg/L each of nonylphenol [1.2], and 17β-estradiol [1.1] in methanol. One hundred microliters of this mixture was injected as with the samples to determine reference retention times of these pertinent OWCs during the HPLC fractionation of active SPE fractions.

Once fluorescence and estrogenic activity chromatograms were obtained, estrogenic bands were identified and the active fractions collected for chemical analysis in repeat HPLC fractionations. Whole bands were collected and reduced under N_2 at <30°C, then transferred to 600 μL amber microvials where they were reduced further and left in a desiccator overnight to remove any residual water.

50 µL of anhydrous pyridine and 50 µL of fresh BSTFA:TMCS (99:1) were added and the vials were capped. Optimal derivatization conditions for suspected OWCs were determined to be 3 hours at 90°C (Ikonomou et al. 2007). One microliter injections were run on a Thermo Finnigan Trace GC/MS 2000 Series (i.e. low resolution MS) with a DB5-HT 15 meter (with a 1 meter polysiloxane guard column) using a temperature program of 100°C held for 3 minutes followed by an 8°C/min ramp to 250°C, then a 10°C/min ramp to 325°C for a final hold of 5 minutes. The injector temperature was 310°C, and the carrier gas was He with a 0.9 mL/min flow. The interface temperature was 250°C, the source temperature was 200°C, and the MS was run in full scan mode (100-520 m/z).

2.2.5 Data analysis

All data analysis was performed using Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA). Full scan GC-MS mass spectra were searched using the NIST 1998 Library, and matched based on the normal identity search algorithm used by Xcalibur 1.4 (Thermo Electron Corporation, Waltham, MA). Matches were performed based on the highest similarity index (SI) value provided by this software.

2.3 Results

2.3.1 Occurrence of 30 wastewater-derived contaminants in 5 WWTPs

30 OWC of concern were analyzed for all the wastewater samples collected in this work (See Table 2-3 for list of compounds analyzed). The results are summarized in Figure 2-2 'a-c' for municipal influents and effluents, and BKME samples. Additionally, all the data summarized in theses figures are available in Appendix IV for each sample of each of the five plants taken in this work. As seen in Figure 2-2b, the greatest levels of steroidal estrogens in wastewater effluent were E1 [1.4] > E2 [1.1] > E3 [1.5]. However, the synthetic steroidal estrogens and progesterones surveyed showed much greater ranges with maxima over 200 ng/L in replicate samples. The synthetic birth control hormone 19-norethindrone [2.1] was particularly high in the influent samples in several cases (Figure 2-2a).

Nonylphenol [1.2] and DEHP [2.18] were found to be the highest non-steroidal synthetic compounds surveyed in both municipal influent and effluent samples (Figures 2-2 a and b). Coprostan-3-one [2.15] and cholesterol [1.16] were the highest fecal sterols, whereas fucosterol [2.16] > beta-sitosterol [1.9] > stigmasterol [2.9] where the highest plant sterols in municipal influent and effluents measured. BKME showed low but significant levels of estradiol [1.1] and pinosylvin [1.8] and relatively high levels of plant sterols, particularly stigmastanol [2.10] (Figure 2-2c) which occurred at a much lower level in municipal effluents (Figure 2-2b). Cholesterol [1.16] and coprostan-3-one [2.15] were nearly absent in BKME, whereas they made up the largest OWCs surveyed in this work for municipal effluents.

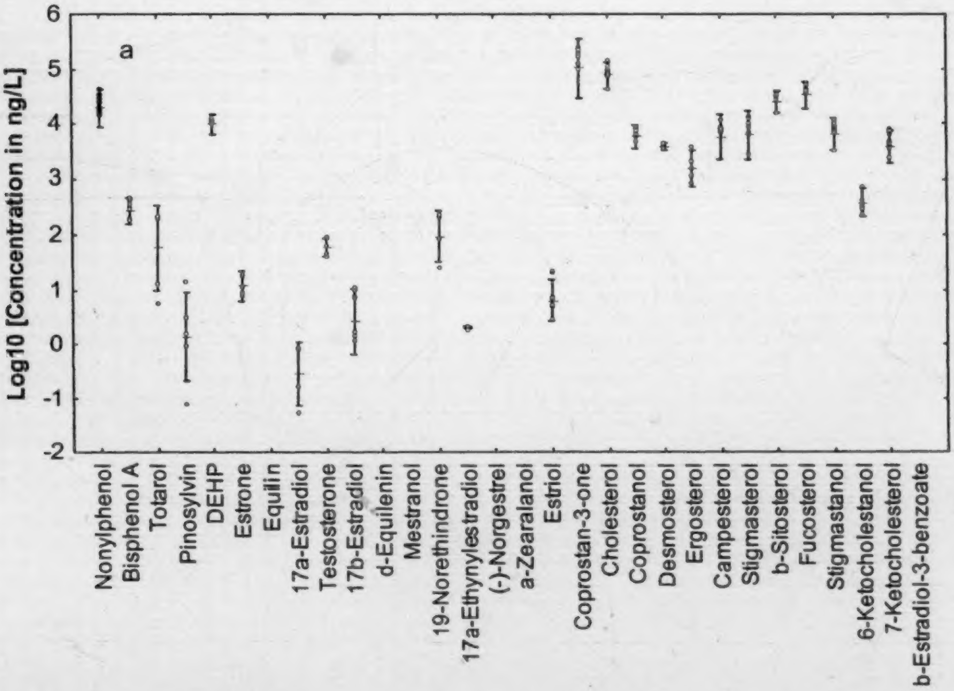


Figure 2-2. Log10 [ng/L] transformed, procedural blank subtracted concentrations of the 30 OWC measured for 5 different wastewaters surveyed (Municipal n_{eff}= 29; n_{inf}= 11; BKME n_{eff}=6). NOTE: a - municipal Influent; b - municipal effluent; c - bleached kraft mill effluent; open circles are raw data points; error bars are ± 1 standard deviation.

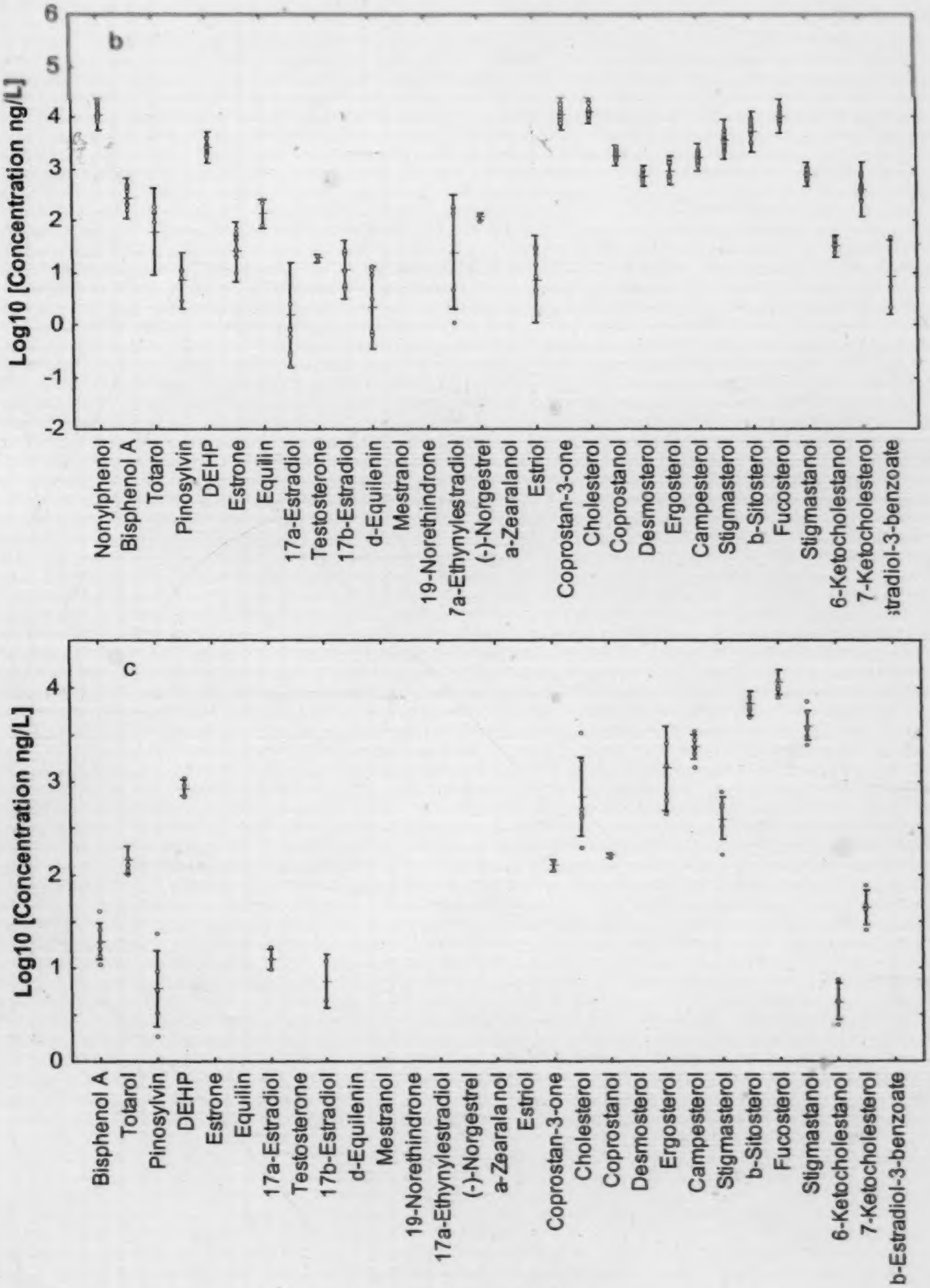


Figure 2-2 (Continued)

Reproducibility in percent relative standard deviation (%RSD) for the influent and effluent samples analyzed was between 5-20% for most compounds (n=5). The %RSD was higher for certain problematic and/or low level compounds including pinosylvin and 17β-estradiol [1.1] (~100%), estriol [1.5] (~66%), ergosterol [2.12] (~61%), d-equilenin [1.7] (~38%), and 7-ketocholesterol [2.14] (~35%). Most compound recovery was between 60 and 120%, whereas lower recoveries were obtained for pinosylvin [1.8], d-equilenin [1.7] and estriol [1.5] as determined by spiked secondary effluent trials. Method detection limits (MDLs) were determined to be 1 ng/L to 0.5 μg/L based 40 mL samples (n=3) (see Appendix V for specific MDL averages and ranges for each compound).

2.3.2 Recombinant yeast assay results

The *in vitro* yeast screen was used to measure the estrogenic response of the whole wastewater samples, as well as the individual SPE fractions of the wastewater samples which were produced at the beginning of the TIE scheme (see Figure 2-3a-c). A greater estrogenicity was observed in the whole wastewater samples relative to the sum of all SPE fractions seen in Figure 2-3. This may come about due to a recovery loss of estrogenic substances during the SPE extraction procedure and/or estrogenic substances which were either too polar or large to extract onto solid phase columns, deviations in the actual DRC of the mixtures which may cause a loss of proportionality in E2 equivalents for the measured compounds (see Appendix XVII for a detailed explanation of this phenomenon), or finally due to the presence of synergistic effects in the whole effluent. Using correlation analysis between SPE fractions and whole effluent estrogenicities it seems that the estrogenic responses of fraction 3 and 4 correlated well to the whole fraction estrogenicity in municipal effluents (r = 0.90 and r = 0.91, $p<0.05$; n=9).

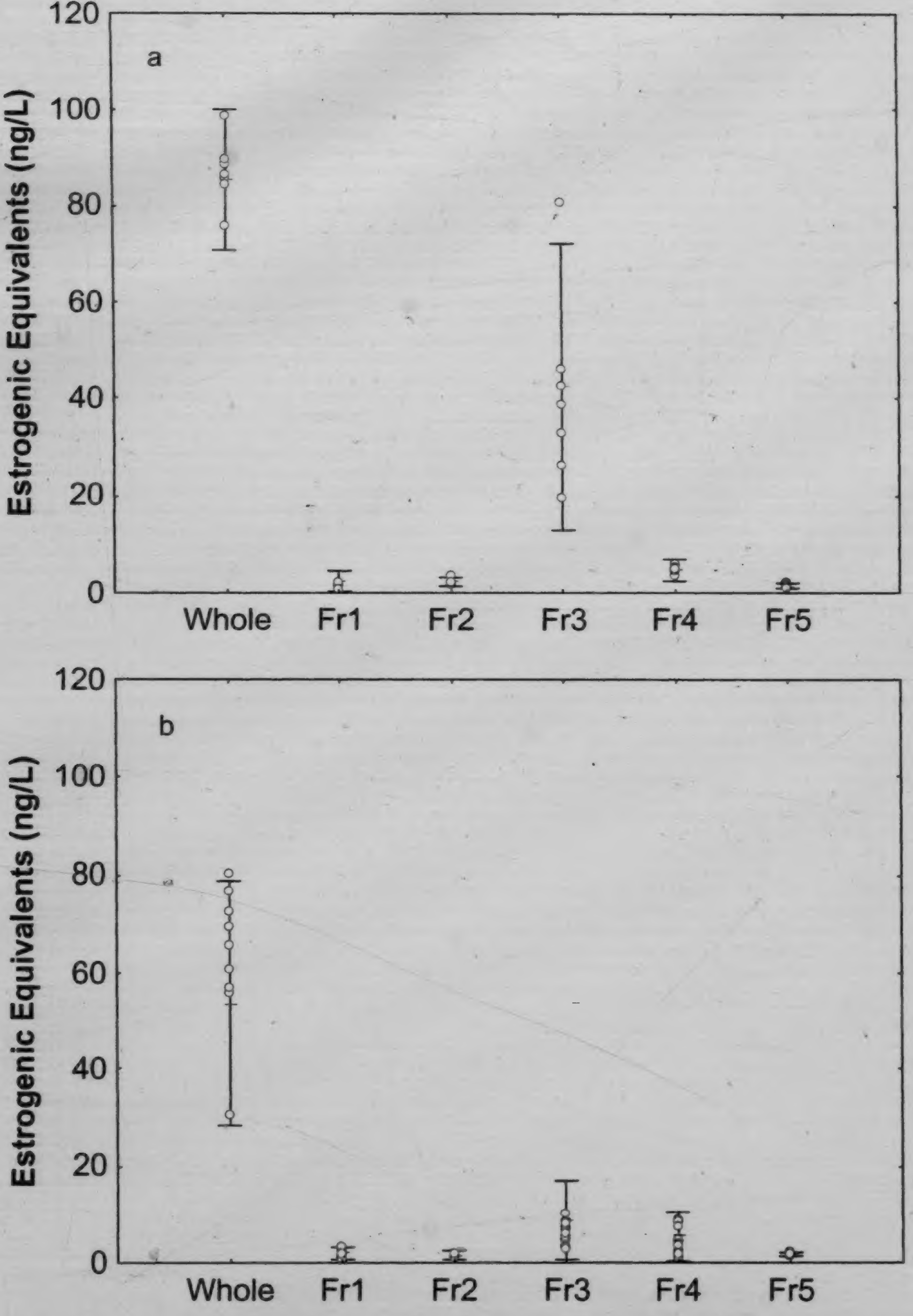


Figure 2-3. ***In vitro*** **estrogenicity results for whole wastewaters and SPE fractions 1-5 for 5 different wastewaters surveyed (Municipal n_{eff}= 11-27;n_{inf}= 9-11; BKME n_{eff}=4-6). NOTE: a - municipal Influent; b - municipal effluent; c - bleached kraft mill effluent (BKME); open circles are raw data points; error bars are ± 1 standard deviation.**

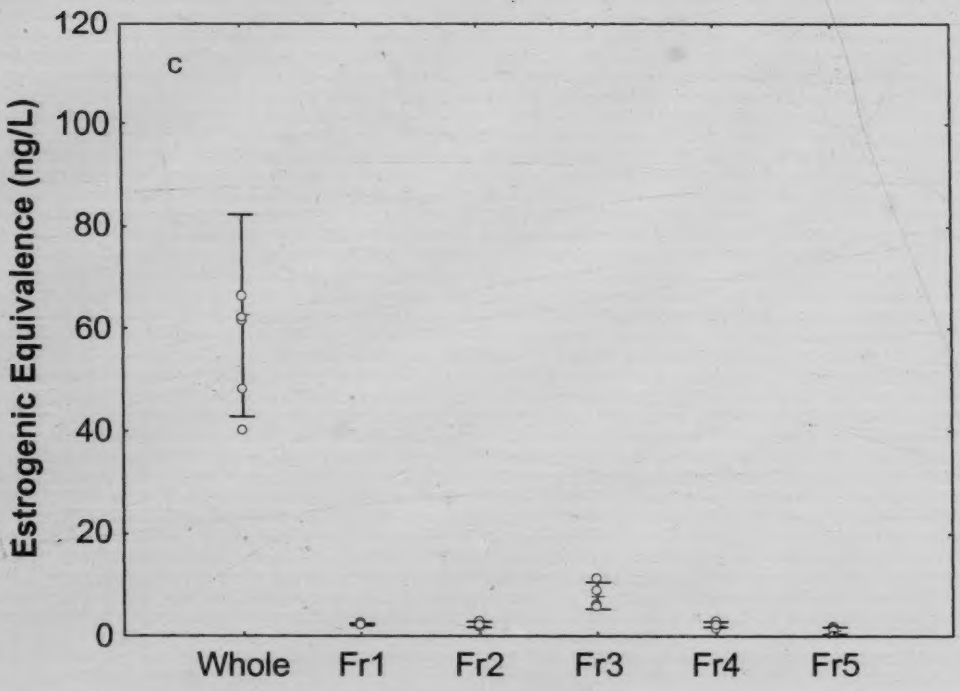


Figure 2-3 (continued)

Using five labelled standards spiked into 200 mL aliquots of site B's effluent (n=3), it was found that 17β-estradiol [1.1] (log $K_{ow} = 3.9$) and bisphenol A [1.3] (log $K_{ow} = 3.4$) eluted predominantly in SPE fraction 3 (eluted with 100% Methanol) whereas, nonlyphenol [1.2] (log $K_{ow} = 4.5$), di-n-octyl phthalate [2.19] (log $K_{ow} = 9.2$), and cholesterol [1.16] (log $K_{ow} = 8.7$) eluted predominantly in fraction 4 (eluted with diethyl ether). None of the tested analytes eluted in fraction 5 (eluted with hexanes) (see Appendix V for summary of SPE trial results). Fractions 3 and 4 did not correlate significantly to the whole influent or BKME estrogenicity (see complete data set in Appendix IV). Based on the whole sample *in vitro* estrogenicities, municipal wastewater influent was more estrogenic than both municipal effluents and BKME (independent sample t-test $p<0.02$), however BKME and municipal effluents did not show statistically significant differences in estrogenicity (independent sample t-test $p= 0.46$).

2.3.3 *Relationships between chemical and in vitro data*

Chemical levels of compounds detected in significant amounts were converted to estrogenic equivalents (EEqs) using published estrogen equivalent factors for the RYA used in this work. These compounds include 17β-estradiol [1.1] (1.000), 17α-ethinyl estradiol [1.6] (0.888), estrone [1.4] (0.096), estriol [1.5] (0.0063), 17α-estradiol [1.1] (0.0525), nonylphenol [1.2] (0.00005), bisphenol A [1.3] (0.00005), (-)-norgestrel [2.2] (0.000004) (Coldham et al. 1997). See Appendix IV for the chemical EEq calculated for each wastewater sample. Positive correlations ($r=0.75$, $p=0.03$ for influent; $r=0.73$, $p=0.06$ for effluent) were found between the sum of estrogenic equivalents from these targeted compounds and the net estrogenic activity measured in the whole effluents for site B samples (Fernandez et al. 2007a). A similar positive trend between calculated chemical EEq and *in vitro* measured E2-Eq was seen for the three effluent samples taken weekly from the AL system (Site E; $r= 0.998$, $p = 0.03$; see Appendix IV for raw data and Appendix XVI for graphical representation). Additionally, the effluent temperature increased with both chemical and *in vitro* E2-Eq from eff1 (6°C), eff2 (8.4°C), and eff3 (15°C) at this plant due to the onset of seasonal warming during the sampling period. Ultimately, the most predominant trend between estrogenic activity in fraction 3 and individual OWCs levels was a negative linear trend between the Log of cholesterol [1.16] and fucosterol [2.16] and estrogenic activity for all the wastewater samples ($r = -0.56$, $p = 0.0002$ and $r = -0.47$, $p = 0.002$, respectively). However, most correlations between chemical and *in vitro* E2-Eq were weak at best ($p>0.05$ in most cases), particularly when examining the 100% MeOH (fraction 3) estrogenic level, likely due to the expression of the estrogen agonists being obscured by high concentrations of human estrogen receptor (hER) antagonists such as bisphenol A [1.3] (as discussed later). Figure 2-4, illustrates a mixed mode relationship between bisphenol A [1.3] concentrations found in municipal effluent samples and corresponding estrogenicity of the semi-polar fraction 3 (100% methanol). The negative linear trend followed by lack of any apparent positive or negative linear trend above fraction 3 estrogenicity of 10 ng/L has been illustrated with a hatch trend line which serves as a visual aid only and not a mathematical model. The inserted figure in Figure 2-4, represents pure compound dose response curves for both E2 and bisphenol A [1.3], in order to illustrate that bisphenol A [1.3] behaved as a xenoestrogen as a pure compounds and was not cytotoxic at high concentrations.

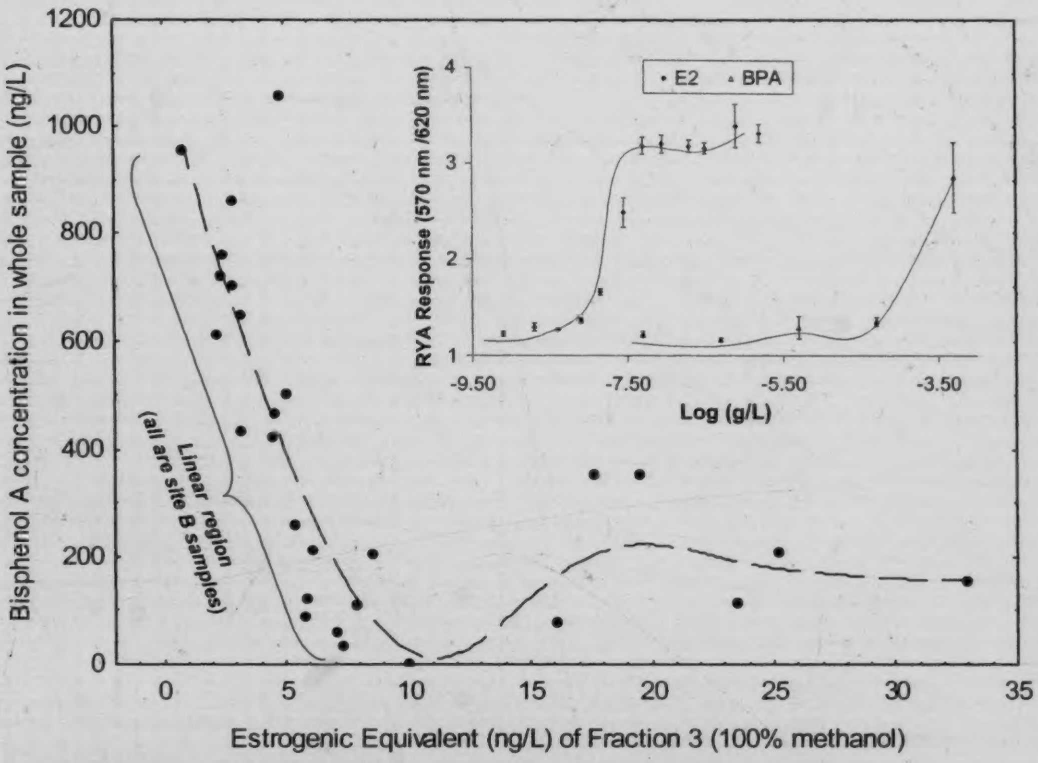


Figure 2-4. Bisphenol A [1.3] concentration in whole effluents versus estrogenic activity of semi-polar SPE fraction (fraction 3) in municipal wastewater effluents surveyed (i.e. excluding BKME). Insert: RYA dose response curves for E2 [1.1] and BPA [1.3] (n=3); error bars are ± 1 standard deviation.

2.3.4 HPLC fractionation of Active SPE

The SPE fractions tested for *in vitro* estrogenic activity and elution of representative labeled chemical species illustrated in Figure 2-3 and specified in section 2.3.2, respectively, formed the initial stage of the modified TIE approach. The second tier involved HPLC fractionation of the most potent of these SPE fractions. More emphasis was placed on the municipal effluent samples due to their greater direct impact on the receiving environment and target organisms based on measured levels of estrogenicity and larger flow into the receiving environment compared with most industrial effluents. Fraction 3 (100% Methanol) was found to contain the majority (46 to 81%) of the estrogenicity detected in the SPE fractions of the effluents. HPLC was used to further fractionate the SPE fractions into finer polarity fractions. An online fluorescence detector and the RYA were simultaneously used as detectors on the HPLC. Active HPLC bands were identified by high estrogenic activity using the RYA and subsequently collected and analyzed by GC-MS (i.e. low resolution) in full-scan mode. GC-MS run in full-scan allowed further separation and identification of the substances present in these active HPLC fractions.

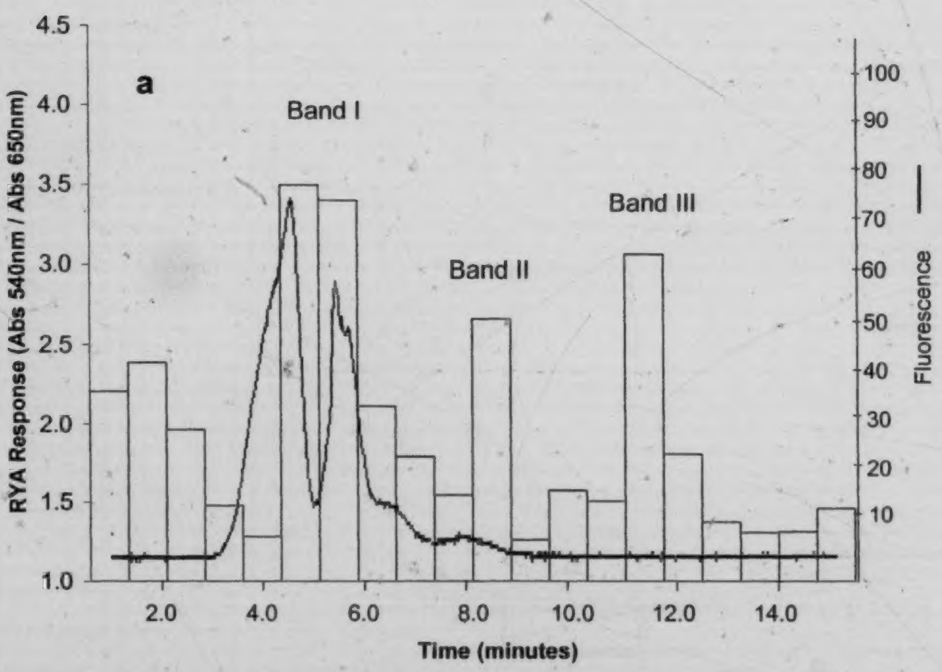
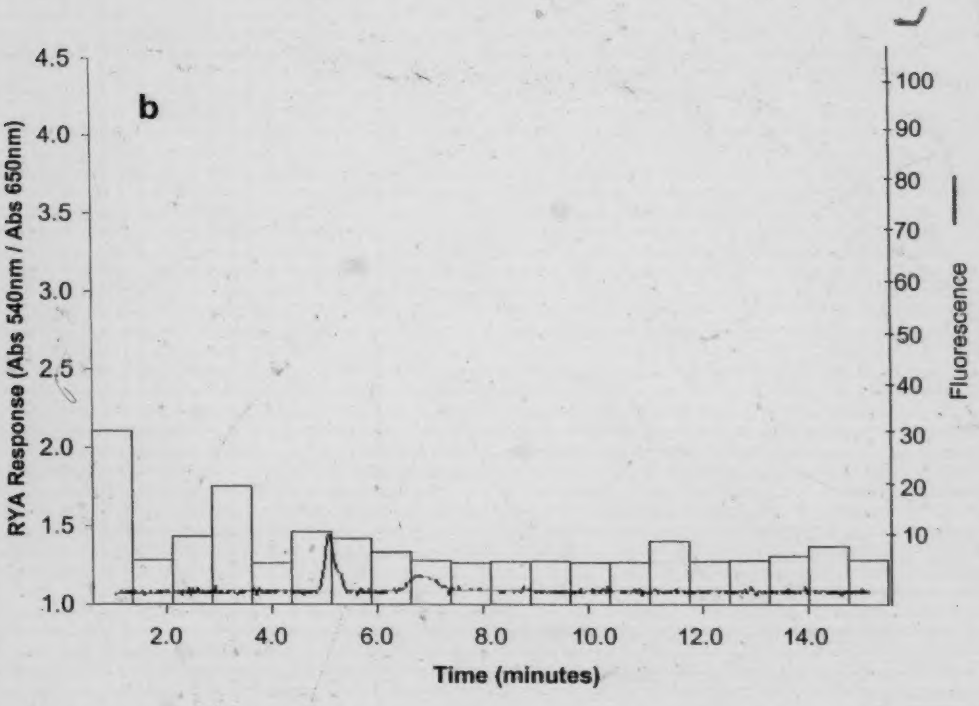


Figure 2-5. RYA activity (shown in bars) and fluorescence (excitation = 225 nm and emission = 295 nm; displayed in % of maxiumum observed standard) chromatograms for a 500 μL injection of (a) site B effluent SPE fraction 3 (100% methanol) and (b) reagent water blank SPE fraction 3 (100% methanol); and 100 μL injection of (c) standard mixture of β-estradiol [1.1] (1,500 ppm) at 4.9 min(1), cholesterol [1.16] (70 ppm) at 5.3 min(2) and 4-nonylphenol [1.2] (1,500 ppm) at 5.9 min(3).



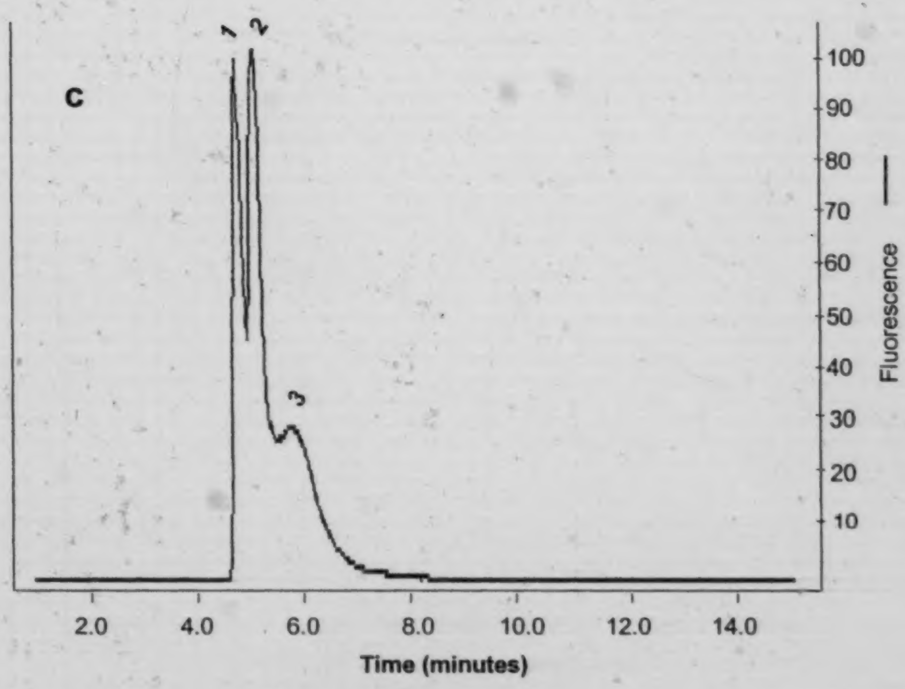


Figure 2-5 (Continued)

Figure 2-5 shows typical results obtained in this fractionation procedure with both fluorescence and RYA response as detectors, illustrating the predominant bands of estrogenicity which were obtained for the 100% methanol SPE fraction of a site B effluent sample (a), versus an appropriate blank (b), along with a 3 standard mixture (fluorescence only) (c). All municipal effluent samples surveyed (site B, C and D) showed a predominant band of estrogenicity around 4.5 minutes, with the example shown in Figure 5 exhibiting additional bands at 8.25 minutes and 11.25 minutes. The ubiquitous band at 4.5 minutes corresponded to the region where 17β-estradiol [1.1], cholesterol [1.16] (i.e. a fluorescently detectable surrogate for sterols) and to a lesser extent, nonylphenol [1.2] standards eluted (Figure 2-5c). However, SPE fraction 3 (100% MeOH), as mentioned previously, does not contain the majority of the cholesterol [1.16] or nonylphenol [1.2] in those samples, and thus the estrogenicity of band I is likely due to steroidal estrogens such as 17β-estradiol [1.1] and similarly eluting compounds based on similarity of retention.

Several of the active fractions were collected over triplicate runs (500 uL injections) to provide sufficient material for GC-MS analysis. Once prepared for gas chromatography analysis, collected HPLC fractions were run via GC-MS as trimethylsilyl (TMS) derivatives for enhanced detection limits for substances with active functional groups including steroid and sterol compounds. The results of the GC-MS analysis and identification of predominant peaks are shown in Table 2-4. The levels of the most abundant substances which were likely contributors to the observed estrogenic activity are summarized here. The identification of these substances is based on full scan fragmentation comparisons to 70 eV spectra in the NIST mass spectral database using the normal identity forward search similarity index. This value is related to the absolute value distance between the reference spectrum and unknown, considering all peaks in the reference library thus allowing for noise in the unknown library (Stein and Scott 1994).

Table 2-4. Summary of peaks identified via GC-MS from estrogenic HPLC fractions from site B effluent SPE fraction 3 (in order of abundance).

Retention Time (min)	Abundance (peak area)[a]	Compound ID (NIST Library)	SI[b]
		Band I	
22.43	21.5x10^{6}	β-Sitosterol [1.9] - TMS	755
22.59	8.6x10^{6}	3β-stigmasta-5,25-dien-3-ol [2.20] - TMS	709
8.64	5.4x10^{6}	Galactouronic acid [2.21] - TMS	547
21.88	3.4x10^{6}	Stigmasterol [2.9] - TMS	785
20.54	1.8x10^{6}	Cholesterol [1.16] - TMS	695
19.64	0.9x10^{6}	Binaphthylsulphone [2.22]	617
		Band II	
7.56	2.5x10^{6}	Terephthalic acid [2.23] - TMS	935
14.62	2.2x10^{6}	Bis(2-ethylhexyl) phthalate [2.18]	920
7.87	1.1x10^{6}	Guaifenesin [2.24] - TMS	743
9.02	0.3x10^{6}	Dibutyl phthalate [2.25]	615
		Band III	
7.66	0.7x10^{6}	Azelaic acid [2.26] - TMS	678
16.73	0.2x10^{6}	D-(+)-Sucrose [2.27] - TMS	624

[a]Peaks >1x10^{6}. [b]Similarity Index based on 1998 NIST Mass Spectral Library.

2.4 Discussion

This work represents an assessment of the occurrence of estrogenic contaminants, other related organic wastewater contaminants and estrogenic activity in selected wastewaters from western Canada including industrial and municipal sources. An adapted TIE procedure was applied to investigate the link between the observed levels of estrogenic contaminants and response of a commonly used recombinant yeast screen. The levels of 30 pertinent wastewater derived estrogenic contaminants and related compounds were measured and reported in samples of municipal influent and effluent, and BKME. In this work, all the results of 5 different treatment plants are summarized in Figure 2-2(a-c) whereas sample specific data is given in Appendix IV. The summary serves as an overview of substances in these wastewaters to help prioritize which compounds may be most problematic throughout municipal wastewater treatment and to the receiving environment in Canada. Natural and synthetic estrogens/progesterones were detected in wastewater samples at levels over 200 ng/L, although the natural estrogens were generally <50 ng/L. Johnson and Sumpter 2001 reported that typical effluent E1 [1.4], E2 [1.1] and E3 [1.5] levels were 5, 1.5 and 20 ng/L respectively. More recently, Servos et al. 2005 reported mean concentrations of E2 [1.1] and E1 [1.4] in influents of 15.6 ng/L (2.4–26 ng/L) and 49 ng/L (19–78 ng/L) and in effluent of 1.8 ng/L (0.2–14.7 ng/L) and 17 ng/L (1–96 ng/L) in a survey of 18 municipal WWTPs across Canada. Our work shows that these compounds occurred well within these ranges for the plants surveyed in this work, but that in some cases the levels increased through the biological treatment process.

Many natural and synthetic steroidal estrogens are excreted by humans as polar conjugates with glucuronic acid [1.11] and sulfate [1.12] (Ternes et al. 1999). Cleavage of these conjugates by extracellular enzymes (e.g. glucuronidase) would result in an increase in free steroid, barring their appreciable degradation or conversion. Our study suggests that some of the plants surveyed showed poor removal, and in most cases in-plant production of natural estrogens was observed. Site B (TF/SC) and D (Aerated Lagoon) showed net increases in E1 [1.4] and E2 [1.1] throughout the biological treatment step (see Appendix IV for plant specific values). Servos et al. 2005 reported that 2 of the 4 lagoons surveyed for E1 [1.4], E2 [1.1] and estrogenic activity showed increased levels of *in vitro* measured estrogenicity after treatment. Additionally, the TF/SC plant surveyed in Servos et al. 2005 showed a similar increase of estrogenicity, accompanied by an increase in effluent E1 [1.4] and E2 [1.1] concentrations.

Synthetic estrogens and progesterones including birth control hormones (mestranol [2.3], 19-norethindron [2.1], EE2 [1.6], (-)-norgestrel [2.2]) and hormone replacement therapy drugs (equilin [2.4], d-equilenin [1.7]) occurred less frequently than their natural counterparts. 19-Norethindrone [2.1] was the most frequently detected and abundant of all the synthetic estrogens/progesterones in the municipal influent. This substance is the primary active ingredient in the popular birth control brand Canada Select™ 1/35 which contains 1 mg 19-norethindrone [2.1] and only 0.035 mg of EE2 [1.6] per tablet. On the other hand, EE2 [1.6] was the most frequently detected synthetic estrogen in the municipal effluents, although it occurred at or below 5 ng/L with some sporadic occurrences of up to 178 ng/L. Johnson et al. 2005 reported a mean of 1.1 ng/L (0.8–2.8 ng/L) in effluents for 17 municipal WWTPs in Europe. Whereas, Drewes et al. 2005 presented EE2 [1.6] concentrations at a maximum of 4.1 ng/L in secondary effluent from 7 United States WWTPs producing water for reuse. Thus, the levels reported in this work are amongst the highest reported in the literature, particularly those occurring in site B's effluent.

Other natural and synthetic substances of concern due to reported *in vitro* estrogenicity, capacity to elicit adverse reproductive effects in chronically exposed aquatic organisms and/or which may be acutely toxic at environmentally relevant concentrations include NP [1.2], BPA [1.3], DEHP [2.18], coprostanol [1.15], and β-sitosterol [1.9] (Jobling and Sumpter 1993; Alexander et al. 1988; Jobling et al. 1995; Gagne et al. 2001; Tremblay and Van der Kraak 1999). Based on the two AST plants, one TF/SC plant and one aerated lagoon surveyed in this work, the observed influent-to-effluent reduction was estimated at 27% for DEHP [2.18], 56% for NP [1.2], 70% for BPA [1.3], 72% for coprostanol [1.15] and 83% for β-sitosterol [1.9] during treatment (see estimated %reduction rows in Appendix IV). NP [1.2], DEHP [2.18] and BPA [1.3] occurred at average levels of 6.8 (1.6-17) μg/L, 6.2 (0.9-17) μg/L, and 0.14 (ND-0.44) μg/L, respectively, in the four municipal wastewater treatment plant effluents surveyed. Not surprisingly, these were some of the more frequently detected OWCs in surface waters from a network of 139 streams across 30 states during a study conducted in 1999 and 2000 (Kolpin et al. 2002). The NP [1.2] concentrations measured in our work were much greater than those reported in a survey of 14 European municipal

WWTPs, where the median concentration was 0.31 μg/L and values ranged from 0.05 to 1.31 μg/L (Johnson et al. 2005). The 96 hour LC_{50} for *Pimephales promelas* (fathead minnow) was 130 μg NP [1.2] /L (reviewed in Kolpin et al. 2002), which is less than 8 times the maximum value observed in our effluents. Furthermore, Arsenault et al. 2004 found that pulse waterborne exposures of 4-NP [1.2] at 20 μg/L were enough to adversely affect the growth and plasma IGF-I (many of the effects of GH are indirectly mediated by this insulin-like growth factor) of Atlantic salmon (*Salmo salar L.*). This, together with the result found in our work, suggests that there may be a potential risk to juvenile salmon exposed to high concentrations of wastewater effluents in the Fraser River. However, further analysis of risk would require taking into account the seasonally variable hydrologic conditions and contaminant dispersion in the receiving environment.

Higher levels of animal sterol (i.e. cholesterol and derivatives) are expected in municipal wastewater due to the large human waste component in this waste stream. Alternatively, plant sterols are expected to be higher in the BKME due to their presence in the wood extracts which are released during processing of pulpwood for pulp production. In particular, adverse effects in aquatic organisms have been reported from exposure to β-sitosterol [1.9] and coprostanol [1.15], both of which were surveyed in this work. β-sitosterol [1.9], associated with bleached mill effluents (i.e. BKME), has been implicated in elevated vitellogenin and reduced pregnenoleone and cholesterol levels in sexually immature rainbow trout in a 21-d in vivo exposure experiment (Tremblay and Van der Kraak 1999). Coprostanol was shown to be estrogenic to freshwater mussels, as measured via vitellin induction upon exposure and thus, is a potential reproductive disruptor (Gagne et al. 2001). Although, both coprostanol [1.15] and β-sitosterol [1.9] showed high influent-to-effluent reduction rates in this work (71-82%), the effluent levels for these compounds still averaged around 1.3 μg/L and 5.5 μg/L for municipal wastewater and 0.2 μg/L and 7.2 μg/L for the BKME, respectively.

β-sitosterol was found to significantly induce vitellogenin in rainbow trout at exposure concentrations starting at 75 μg/L.

The *in vitro* estrogenic activity in the predominant estrogenic SPE fraction (fraction 3) was reduced by an average of 64% in site B, C and D's effluent, but increased slightly in site E's effluent, compared to the influent sample. Whole effluent estrogenicity ranged from as low as 9 ng/L E2-equivalents in site E's effluent to 98 ng/L E2-equivalents in site A's effluent. A considerable amount of temporal fluctuation was apparent in estrogenic activity for site B as assessed in eight weekly samples (see raw data in Appendix IV). The estrogenic activity in these effluents ranged from 56 to 80 ng/L and thus, time of sampling will significantly influence the estrogenic activity reported for a particular plant's effluent. The estrogenic activity in the samples were greater than those published in the literature, which were generally <20 ng/L for municipal wastewater effluents (Kirk et al. 2002; Svenson et al. 2003; Holbrook et al. 2002). Reasons for this large discrepancy may include differences between the methodology adopted for the present study, and those employed previously by other researchers. In the present work, the samples were filter sterilized within 48 hours of collection, and particulate bound estrogenic activity was included, as was any estrogenic material potentially lost in the filter sterilization process (see methodology). The results in the literature were all obtained from extracted samples (i.e. SPE or liquid-liquid), were not recovery corrected, and may not have included particulate bound estrogenic activity. Ultimately, the method of analysis, including whether the estrogenic activity was based on whole effluents or on extracts, may result in differences in magnitude among different studies and thus, should be considered when making such comparisons.

Several significant relationships between targeted chemical analysis and *in vitro* results were revealed in this work. The first one, which has been already discussed in Fernandez et al. 2007a, was a significant linear correlation between estrogenic activity and the sum of estrogenic equivalents for significant environmental estrogens quantified in site B's influent samples. Inverse relationships between cholesterol [1.16] and fucosterol [2.16] with wastewater estrogenicity were revealed in this work. Additionally, BPA trends with effluent estrogenicity (SPE Fraction 3) suggested that components in the matrix were having an anti-estrogenic or suppressive effect within the *in vitro* screen. BPA [1.3] was tested alone and identified as a positive hER agonist within the *in vitro* screen used in this work (Figure 2-4 insert) and by other researchers (Coldham et al. 1997). Thus, the decreased estrogenic response in the mixtures where higher amounts of BPA [1.3] were detected compared to those where lower concentrations were detected may be attributed to an anti-estrogenic effect in the mixtures. This effect was tested *in vitro* by spiking 3 fraction 3 mixtures (two samples from site B and one from site D) in triplicate with 3 levels of BPA [1.3] (0.5, 1.0, 1.8 μg/L) including unspiked controls. The response of the spiked mixtures was not statistically lower than the unspiked mixtures by a narrow margin (randomized complete block design, $p = 0.1$; see Appendix VII for raw data). Thus, it could not be confirmed that BPA [1.3] at higher concentrations and in the presence of natural estrogens was acting as an anti-estrogen.

The other interesting relationship already mentioned was that samples having higher fucosterol [2.16] (2nd most abundant OWC surveyed) and cholesterol [1.16] (3rd most abundant OWC surveyed) concentrations showed lower estrogenic activity, implying that wastes of higher organic strength may show lower estrogenic activity via the RYA. A possible explanation for this observation is that cholesterol and fucosterol [2.16] levels may correlate with the levels of other toxic substances, which may suppress the response of the RYA. Ultimately, these trends illustrate that although the RYA is very useful in identification of positive estrogen agonists, and quantifying estrogen activity in the absence of suppression / anti-estrogenic effects, negative RYA results should always be accompanied by chemical quantification and identification of potential estrogenic species.

The potential exists for other unidentified or un-quantified hER agonists to be present in the wastewater. Identification and quantification of the effects of such compounds would strengthen the relationship between the targeted chemicals and estrogenic activity, and would ultimately facilitate predicting the impact on aquatic organisms and downstream source water supplies. The RYA was used to guide a two-tiered fractionation of the solid phase extracted wastewater for these purposes. HPLC fractionation of the active SPE extracts may resolve further estrogenic bands for subsequent identification, as well as provide a method to potentially detect estrogens in the absence of suppressive or anti-estrogenic components. Figure 2-5a, shows three distinct bands of estrogenicity which were resolved using online RYA with fluorescence detection. Band I eluted in the same region as E2 [1.1], cholesterol [1.16] and NP [1.2]. Based on the previous observation that the latter two substances would elute primarily in SPE fraction 4, it would appear that the estrogenicity of band I would be caused by E2 [1.1] and/or similarly eluting compounds. However, in order to elucidate the types of substances which eluted in all three estrogenic HPLC bands (Figure 2-5a), the major substances identified by GC-MS in these bands are listed in Table 2-4 for site B's effluent, SPE fraction 3. β-sitosterol [1.9], a major plant sterol was detected in large amounts in band I, and may have contributed to some of the estrogenicity observed in this band, as this substance is a reported weak estrogen agonist (Tremblay and Van der Kraak 1998). Additionally, the plant sterols stigmasterol [2.9] and 3β-stigmasta-5,25-dien-3-ol [2.20] which are oxidized derivatives of β-sitosterol [1.9], along with several fatty acids, galacturonic acid [2.21] and binapthylsulphone [2.22] were detected and identified. Several of the steroidal estrogens from the target list (see Table 2-3) were screened for in the active bands using selected ion monitoring (SIM), but none were found. High concentrations of fatty acids and other natural products were found in the region where several steroidal estrogens would have eluted, and may have masked their detection if any were in fact present at detectable amounts. The chemical identification results for bands II and III contained some of the same components found in band I at much lower levels indicating that they may have trailed into these bands due to their high abundance leading to column overloading (only unique substances are shown for each band in Table 2-4). Terephthalic acid [2.23] was found to elute primarily in band II but trailed considerably into band III as well, and was thus not likely to have been responsible for the resolved bands observed via RYA. Band II contained Bis(2-ehtylhexyl) phthalate (DEHP) [2.18] and dibutyl phthalate (DBP) [2.25], which are commonly reported phthalate esters in the environment (Lin et al. 2003). DBP [2.25] was amongst the selected phthalate esters which exhibit weak ER-mediated activity in some *in vitro* assays assessed by Zacharewski et al. 1998 and thus, may partially explain the estrogenic response of band II. The presence of pharmaceutically active compounds guaifenesin [2.24] (7.87 min in band II), used in cough/sinus preparations and azelaic acid [2.26] (7.66 min in band III) used in acne preparations (Anon. 2007) show that there are significant amounts of personal care products and pharmaceuticals which may potentially have adverse effects in exposed aquatic organism in the receiving environment. However, the focus in this work was on estrogenic substances in Canadian wastewater. Ultimately, our results suggest that the bulk of the estrogenicity in the municipal wastewater which is thought to be due to steroidal estrogens, may also be in part due to high levels of phthalate esters and natural sterols in the wastewater.

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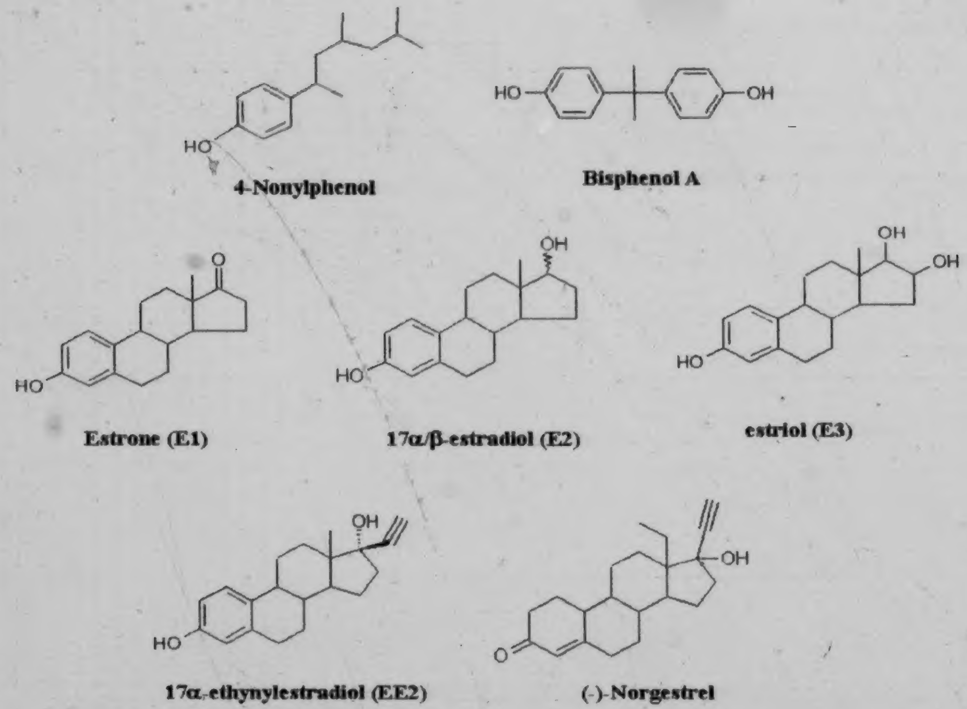
Chapter3- Assessment of environmental estrogens and the intersex/sex reversal capacity for Chinook salmon (*Oncorhynchus tshawytscha*) in primary and final municipal wastewater effluents

This chapter was accepted for publication in *Environment International* on December 13th, 2006 (Fernandez et al. 2007). It presents an assessment of the levels of several pertinent eEDCs in wastewater using a chemical determination method, an *in vitro* bioassay and exposure experiments with a target fish species in western Canada. It represents a complete risk based research program aimed at assessing the threat of reproductive disruption in target species exposed to municipal effluents which are continuously discharged into their environment.

3.1 Introduction

Environmental estrogens are a subset of chemicals which make up a group of environmentally important compounds known as endocrine disrupting compounds (EDCs) and include industrial chemicals, natural and synthetic steroidal estrogens, and various naturally occurring phytoestrogens (Johnson and Sumpter 2001; Kolpin et al. 2002). One of the largest bodies of evidence illustrating endocrine disruption in wild organisms due to an anthropogenic stressor is the scientific literature reporting reproductive effects in several fish species exposed to municipal wastewater effluents and pulp/paper industry discharges (Denton et al. 1985; Jobling et al. 1998; reviewed in Vos et al. 2000). In North America, Nagler and colleagues (Nagler et al. 2001;) found a high proportion of physiological female chinook salmon from three naturally spawning populations in the Columbia River tested positive for a male-specific DNA marker. From these results, the authors hypothesized that endocrine disruption may be playing a role in the sex determination of these fish. Such sex reversal effects have not been observed in British Columbia populations Devlin et al. 2005, but laboratory experiments have provided some evidence for sex reversal in this species arising from exposure to municipal and industrial effluents (Afonso et al. 2002). Thus, a possible mechanism for sex reversal of genetically male salmon involves exposure to environmental estrogens. Domestic and industrial wastewater are well known primary point-source inputs for many contaminants of concern in aquatic environments including EDCs (Liu and Liptâak 1997).

The compounds shown in Figure 3-1 represent typical environmental estrogens which have been identified as likely culprits of reproductive disruption in aquatic organisms in the vicinity of wastewater discharge zones. Nonylphenol (NP) [1.2] the ultimate break-down product of non-ionic surfactants nonylphenol ethoxylates (NPEOs) [1.17] used in domestic and industrial soaps and detergents has shown to be estrogenic via *in vitro* yeast assays, rat uterotrophic assay and exposed fish (Routledge and Sumpter 1996; Odum et al. 1997; Gray and Metcalfe 1997). Bisphenol A (BPA) [1.3] is an environmentally ubiquitous industrial chemical used to make plastic/epoxy resins and other products and a known endocrine disruptor (Krishnan et al. 1993). BPA [1.3] has shown to be acutely toxic to freshwater and marine aquatic organisms at concentrations of 1–10 mg/L (Alexander et al. 1988). However, it is the sub-lethal toxicity of BPA [1.3] which is of greater environmental concern as this substance. BPA [1.3] has been shown to induce ovotestes at concentrations as low as 10 μg/L in Japanese medaka (*Oryzias latipes*) (Metcalfe et al. 2001) and inhibit the development of secondary sexual characteristics in swordtails (*Xiphophorus helleri*) over a concentration range from 0.2 to 20 μg/L (Kwak et al. 2001). Synthetic and biogenic steroidal estrogens in sewage are also of concern due to their high potency and thus capacity to cause adverse effects to the reproductive status of exposed wildlife. The endogenous female estrogens, estrone (E1) [1.4] and 17β-estradiol (E2) [1.1] along with commonly used synthetic estrogens in birth control formulations such as 17α-ethynylestradiol [1.6] (see Figure 3-1) have all been shown to contribute significantly to the estrogenicity of wastewater effluents (Desbrow et al. 1998).



4-Nonylphenol **Bisphenol A**

Estrone (E1) **17α/β-estradiol (E2)** **estriol (E3)**

17α-ethynylestradiol (EE2) **(-)-Norgestrel**

Figure 3-1. Structures and common names for typical environmental estrogens found in domestic wastewater.

In this work, the concentrations of these important environmental estrogens are assessed using a gas chromatography - high resolution mass spectrometry (GC-HRMS) based method. Additionally, in order to capture any estrogens which may not have been included in our list of targets, estrogenic activity was assessed using an *in vitro* recombinant yeast assay (RYA). These analytical techniques were applied to primary and final effluents for one of the largest trickling filtration solid contact (TF/SC) municipal wastewater treatment plants in North America. The TF/SC plant features conventional primary settling tanks, a hybridized trickling filter - activated sludge biological reactor followed by conventional secondary settling and finally tertiary chlorine disinfection. The final effluent with BOD and TSS of ~12 mg/L is discharged into the Fraser River which is frequented by Pacific salmon including Chinook salmon runs. Additionally, during periods of high plant influent flows (i.e. large rain events), it is possible that primary effluent would be bypassed and discharged directly into the river. To determine if estrogens in effluent from the TF/SC plant could alter sex determination in salmon chinook salmon eggs were exposed to several dilutions of the wastewater, including environmentally realistic concentrations, from the eyed stage of development to 28-days post hatch.

3.2 Experimental methods

3.2.1 Sampling

The trickling filter / solid contact (TF/SC) biological secondary treatment with chlorine disinfection had a daily average flow of 455 megaliters per day (MLD), mean hydraulic retention time of 0.3-2 h (bioreactor) and served a suburban population of 740,000. The TF/SC process consists of a high rate attached growth reactor (trickling filter) followed by a short solids residence time aerobic suspended growth reactor (solid contact tank) for the purpose of improving the settling characteristics of biomass wasted from trickling filters as well as providing additional oxidation of carbonaceous organic material (Slezak et al. 1998). Several liter grab samples were taken in the morning at established sampling points (by plant personnel) to provide enough material for chemical, *in vitro* and *in vivo* experiments. The TF/SC plant was sampled weekly from established sampling points directly downstream of the primary sedimentation process and from the final plant effluent each Tuesday for eight weeks from December to February, 2002-03. The weekly effluent samples were split, where a fraction of the sample went for chemical and *in vitro* analysis and a much larger fraction remained in a cold room and was used in the static renewal experiments. In an attempt to minimize the impact of on-going biological and physical (i.e. UV) degradation in the samples, same-day sample collection and extraction was applied whenever possible (however due to shipping time longer delays of up to 48 hours may have occurred during which samples were stored on ice). Finally, aliquots of each sample were frozen at –20°C unpreserved, whereas the bulk (~3 L) was preserved with 5% formalin (chemistry samples only) and stored at 4-6°C for archive purposes.

3.2.2 Recombinant yeast assay (RYA)

The recombinant yeast strain used in this work was obtained from Prof. J.P. Sumpter from Brunel University, Middlesex, UK. This yeast was modified to contain the DNA sequence of the human estrogen receptor (αhER) on the main chromosome as well as an expression plasmid carrying the reporter gene *Lac-Z* which encodes for the enzyme β-galactosidase. The procedure was carried out as specified in Routledge and Sumpter 1996. For calculation of β-galactosidase activity, the ratio of absorbance at 540 nm to that at 650 nm (i.e. optical density) was used. Dose-response curves (DRCs) were plotted as the blank subtracted β-galactosidase activity for twelve estrogen (E2) [1.1] standards run in triplicate verses the log of the concentration (i.e. log [g/L]). The curves were fit using a sigmoidal dose-response curve (variable slope), Marquardt-Levenberg algorithm in SigmaPlot version 8.0 (SPSS, Chicago, IL, USA). Coefficients of determination (r^2) of 0.998 were observed for our dose-response curves. See Appendix III for more details of the RYA protocol and an example of the DRC calculations.

Two hundred milliliters of final effluent or 100 mL of primary effluent was glass wool filtered, a 10 mL aliquot of the filtrate was filter sterilized using 0.2 µm Puradisc® cartridge filters (Whatman, Middlesex, UK) and stored in sterile glass vials for RYA analysis. The glass wool plugs were rinsed with 5 mL of deionized water followed by 5 mL of anhydrous ethanol which was collected for RYA analysis. Additionally, 0.2 µm filters used to sterilize aqueous fractions were rinsed with a few milliliters of ethanol to account for any estrogenic substances lost in filter sterilization. Samples were all run in triplicate wells and in duplicate assays for one of each eight primary and final effluents samples. All values were blank subtracted using a blank of double deionized water treated identically to the samples. The estrogenicity results of the ethanol rinsates were quantitatively combined to that of the filtered liquid to provide an overall response for the whole effluent.

3.2.3 Chemical analysis

The method used to determine the levels of several EDCs in wastewater samples has been previously developed and accepted for publication (Ikonomou et al. 2007). In brief, 40 mL of sample was extracted 3 times with approximately 10 mL dicloromethane (DCM) using sonication. Extracts were reduced under a gentle stream of nitrogen, dried over sparing amounts of sodium sulphate and cleaned up using 5% (w/w) deactivated florisil. Extracts were derivatized using 50 μL of anhydrous pyridine and 50 μL of fresh bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) for 3 hours at 90°C. One microliter of each sample was injected (splitless mode) onto the GC-HRMS which was comprised of a Hewlett Packard 5890 Series II Gas Ghromatograph coupled to a VG AutoSpec magnetic sector mass spectrometer (Micromass UK Ltd., Manchester, UK). Further details of the instrumental method including chromatograms and ions monitored can be found in Figure A2-1 and under the "Instrumental analysis" heading both located in Appendix II. Five non-naturally occurring surrogate standards were used as internal standards for recovery correction and performance evaluation (Appendix II, Table A2-1). Quality control samples including procedural blanks, spikes and duplicates were run with each batch of 10 samples. Results are reported as recovery corrected and blank subtracted values (ng/L). Internal standard recoveries were generally >70% and never outside of 40-120% as a quality control criterion. Further details of the quality assurance / control protocol can been found under the "QA/QC" heading in Appendix II. Linear correlation analysis of the chemically determined estrogenic equivalents (EEq) versus RYA response normalized to E2 [1.1] (E2-Eq) was performed using Statistica 7.0 (StatSoft Inc, Tulsa, OK).

3.2.4 Exposure experiments with chinook

Chinook salmon eggs at the eyed stage of development were transferred to plastic perforated egg incubation boxes (100 eggs per tank, 200 eggs per exposure concentration) and placed in glass aquaria in a temperature-controlled room at

10°C until 28 days posthatch. This exposure period was chosen based on previous work that has shown that the critical period of sexual differentiation in Pacific salmon occurs around the time of hatching (Baker et al. 1988; Afonso et al. 2002). Each aquarium contained 4 liters of test solution and a static renewal system was used with test solutions being completely renewed twice a week. The chinook were exposed to either well water only, or 1%, 10%, or 100% TF/SC primary effluent, or 1%, 3%, 10%, or 100% of TF/SC secondary effluent in duplicate tanks. Effluents were diluted in well water for the exposures. Following the 28-day exposure period the alevins were initially transferred to Heath hatchery trays supplied with well water at 10°C, and then moved to larger tanks supplied with well water at Rosewall Creek Hatchery, Vancouver Island, British Columbia for grow-out until they were sampled on June 20, 2003 (5 months posthatch). Random sub-samples of 30 fish from each replicate tank were removed by dip netting and were sacrificed using an overdose of the anaesthetic MS-222 (tricaine methanesulfonate; Syndel Laboratories, Vancouver, British Columbia, Canada). Blood samples were taken by caudal puncture for analysis of genetic sex, and weights and lengths were recorded.

3.2.5 Molecular biology

For determination of genetic sex a Y-linked marker OtY1 was used (Devlin et al. 1991). Two microliters of blood from each fish was added to 100μl of 0.01M NaOH to lyse cells and yield template DNA. The samples were then frozen at -20°C until they were analyzed. Each sample was boiled for 7 minutes at 100°C to denature the template. The primers used were OtY1 (5'-GATCTGCTGGCTGGATTTGG-3') and OtY2 (5'-CCAGCGATGGTTTGTTTGAG-3'). PCR reactions were performed in a total volume of 30 μl, using 1μl of template DNA, 3 μl of 10x PCR buffer, 3 μl 2mM dNTP, 0.9 μl 50mM $MgCl_2$, 0.6 μl 25mM Oty1/2, 20.95 μl of de-ionized water and 0.15 μl of Taq DNA polymerase. To confirm genetic sex another Y-linked marker, a growth hormone pseudogene (GH-P) was used (Du et al. 1993; Devlin et al. 2001). The primers used for this were GH5 (5'-

AGCCTGGATGACAATGACTC-3') and GH6 (5'-TACAGAGTGCAGTTGGCCT-3') and the PCR reaction was the same except that 1.2 μl of $MgCl_2$ was added, thereby reducing the deionized water to 20.45 μl. The PCR reaction conditions were: initial denaturation of DNA at 95°C for 3 minutes, followed by 35 cycles of amplification (denaturation, 94°C for 1 min; annealing, 52°C for 1 min; and extension, 72°C for 1 min), and a final extension at 72°C for 7 minutes. Samples were analyzed in 2% agarose gels. In each PCR reaction series known male and female positive controls were utilized.

3.2.6 Histology

A whole body cross section was cut from each sampled fish and preserved in Davidson's preservative (300ml ethanol, 200ml 37% formaldehyde, 100 ml acetic acid, and 300 ml deionised water) for histological sex examination. The preserved samples were then transferred to 95% ethanol and subsequently embedded in paraffin. Sections (4-5um) were cut from the whole body cross sections and then stained with hematoxylin and eosin.

3.3 Results and discussion

3.3.1 Environmental estrogens and estrogenicity

The concentrations of several environmental estrogens together with the calculated chemical estrogenic equivalence and measured *in vitro* estrogenicity are shown in Figure 3-2. Overall, these results show that not all environmental estrogens were removed or reduced by the biological treatment (TF/SC) process at this plant. Natural estrogens such as E1 [1.4] increased in the final effluent, likely as a result of the cleavage of conjugated estrogens and/or the well characterized oxidation of E2 [1.1] to E1 [1.4] (Joss et al. 2004).

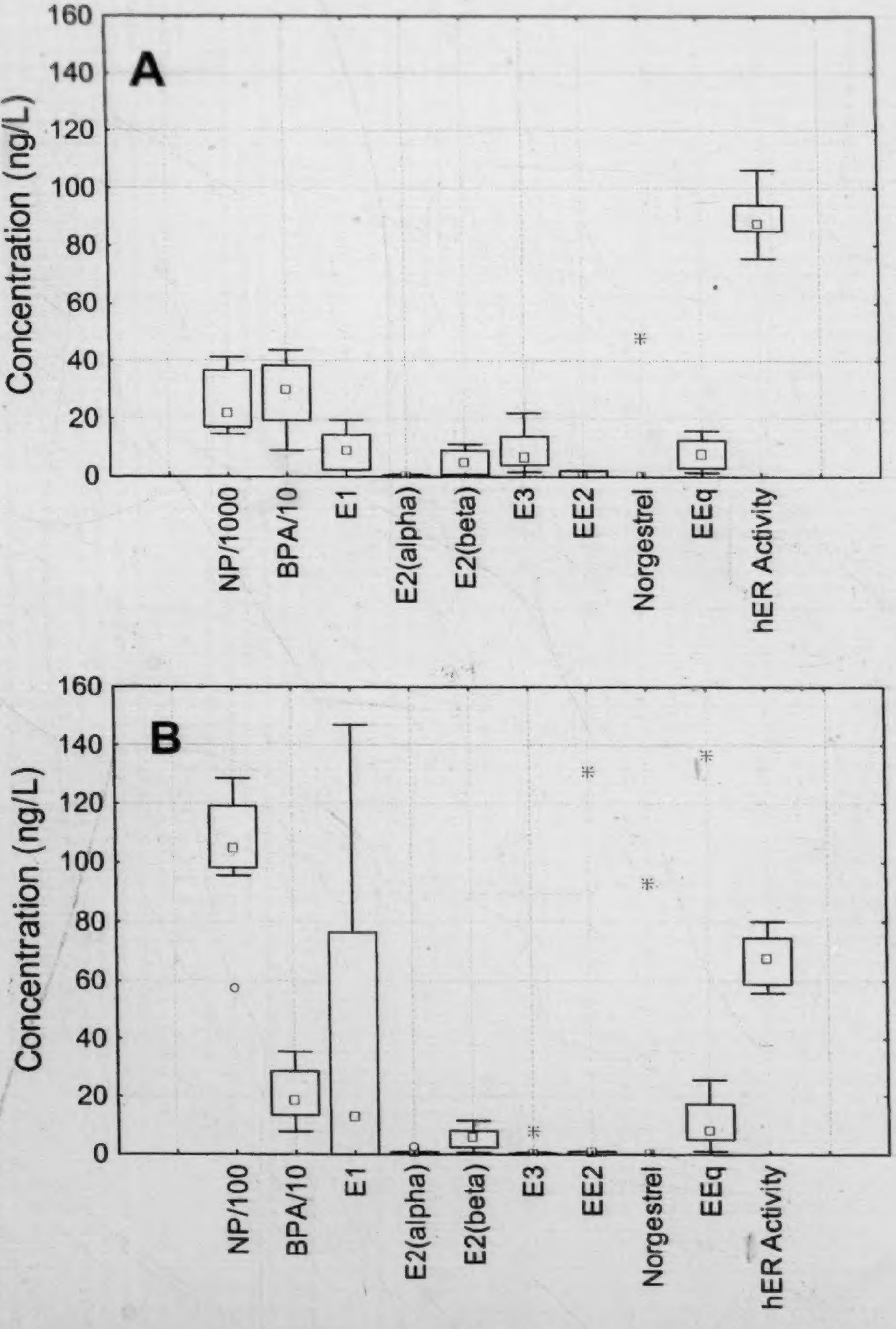


Figure 3-2. Box plot of the pertinent environmental estrogens, chemical estrogen equivalents (EEq) and *in vitro* ER activity (E2-Eq) in TF/SC plant's primary (n=8) (A) and final (n=8) (B) effluents. EEq was calculated based on relative potencies of NP [1.2] (0.005%), BPA [1.3] (0.005%), E1 [1.4] (9.6%), αE2 [1.1] (5.25%), βE2 [1.1] (100%), E3 [1.5] (0.63%), EE2 [1.6](88.8%), Norgestrel [2.2] (0.0004%) (Coldham et al. 1997).

For the most part, the levels of E1 [1.4], E2 [1.1], E3 [1.5] and EE2 [1.6] measured in the samples were well within the typical range observed in municipal wastewater of between 0.5-20 ng/L (Johnson and Sumpter 2001). However, a few of the final effluent samples did show a high concentrations of E1 [1.4] (up to 147 ng/L) which may indicate high organic loadings and the likely cleavage of E1 [1.4] conjugates entering the plant. NP [1.2] levels where 15-41 μg/L in the primary and 6-13 μg/L in the final which was on the low side of the large range (<0.2-2250 μg/L) published for this compound in plant influents and effluents in Europe and the US (Johnson and Sumpter 2001; Birkett and Lester 2003). BPA [1.3] was measured ranges of 88-438 ng/L and 77-353 ng/L in the primary and final effluents in the 8 week sampling period. These data indicate a reduction of approximately 22% by TF/SC secondary / biological treatment for BPA [1.3]. However, activated sludge treatment has been shown to remove up to 99% of BPA [1.3] via biodegradation mechanisms (Birkett and Lester 2003) and thus, the TF/SC system assessed here appears to be less efficient in removing this environmental estrogen. Removal efficiency for all other monitored environmental estrogens in this work was complicated by the fact that several have shown in-plant production.

In this work, the estrogenic equivalence factor or relative potencies for the 8 pertinent environmental estrogens illustrated in Figure 3-2 measured via a similar *in vitro* hER recombinant yeast screen were used to generate a cumulative estrogenic equivalence (EEq). This total chemical EEq for each sample represented, on average, 9 and 37% of the hER activity in the sample for the primary and final effluents, respectively for the entire sampling period. Murk et al. 2002 reported discrepancies as high as 80% in wastewater effluents using an ER-CALUX® bioassay versus chemical analysis determined E2-equivalents. Potential explanations for the large discrepancy between chemical estimation of estrogenicity (assuming an additive response) and observed net estrogenic activity *in vitro* include; a) unidentified chemical species which need to be incorporated into the targeted chemical analysis; b) synergistic effects between estrogens and matrix components which would invalidate the method of additive response used in chemical based estrogenic equivalence (Silva et al. 2002). Due to multiple possible causes that may have contributed to the observed discrepancy in magnitude in EEq and *in vitro* hER response, we have taken advantage of the week-to-week variability in order to estimate how well the chemical EEq correlates to the hER irrespective of the magnitude differences.

Linear relationships between EEq and hER activity were observed for the samples in this work shown in Figure 3-3. These observations support the hypothesis that the targeted chemicals in this work are responsible for some of the estrogenicity in the whole samples assessed. Explanations for the magnitude difference between chemical and *in vitro* measured EEq include, other xenoestrogens present in the mixture not measured chemically or bio-activated *in vitro*, deviations in the actual dose response curves of the test chemicals which may cause a loss of proportionality in E2 equivalents for the measured compounds (see Appendix XVII for a detailed explanation of this phenomenon), and finally, synergistic effects may have been present which would not be accounted for in the chemical EEq calculation. The correlation coefficients (r) were found to be >0.7 for both plots, with a statistically significant positive correlation between EEq and hER activity at 95% and 90% in the primary and final effluents, respectively (Figure 3-3).

Many environmental estrogens, such as ethynylestradiol [1.6], one of the active ingredients in the female birth control pill, have been reported to elicit adverse biological effects in fish at exposure concentrations of as low as 1 ng/L (Jobling et al. 2003). This substance was found to be on average <1 ng/L in effluent samples with the exception of the extreme value of 131 ng/L for this substance found in the week 8 final effluent (this value was excluded from the Figure 3-3B due to its extreme nature). However, the levels of E2 [1.1] averaged 5.5 ng/L in the effluents which should be enough to induce reproductive effects in sensitive species.

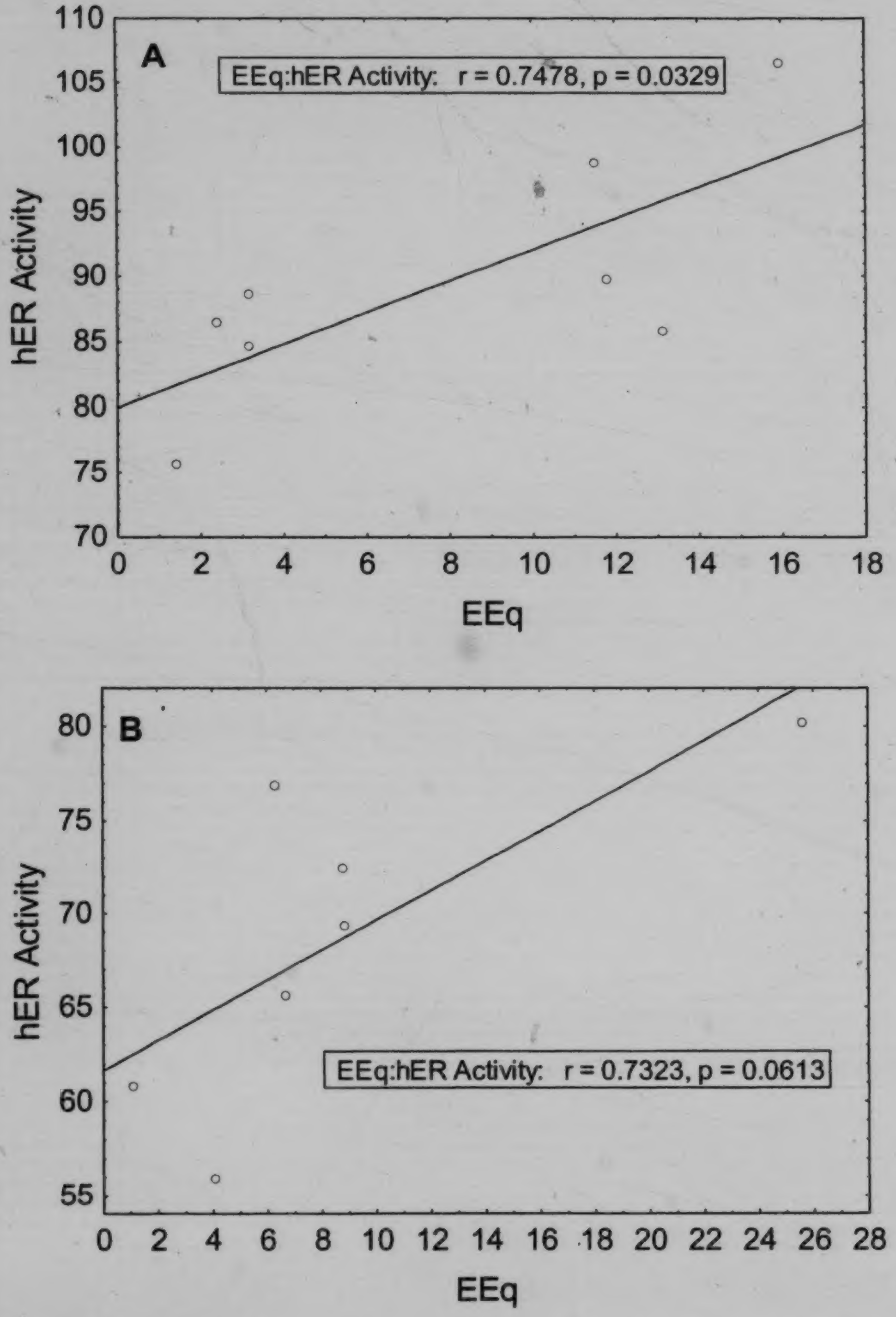


Figure 3-3. Linear correlation results for EEq and hER response in ng/L for primary (n=8) (A) and final (n=7) (B) effluents.

Table 3-1 illustrates the lowest observable effects levels (LOEL) in fish for some of the more abundant environmental estrogens assessed in this work, together with the mean values observed in the effluent samples.

Table 3-1. Reported levels for adverse endocrine effects in fish.

Compound	*Lowest observable effects level (LOEL)*	*Reported effect(s)*	*Mean effluent level*
17α-Ethynylestradiol [1.6]	1 ng/L[a]	↑Vitellogenin	<1 ng/L*
Bisphenol A (BPA) [1.3]	1 μg/L[a]	↓Spermatogenesis	0.2 μg/L
Nonylphenol (NP) [1.2]	20 μg/L[b]	↓Growth ↓Plasma IGF-I	10 μg/L
17β-Estradiol (E2) [1.1]	1-10 ng/L[c]	↑Vitellogenin	5.5 ng/L
Estrone (E1) [1.4]	25-50 ng/L[c]	↑Vitellogenin	41 ng/L

*Excluding extreme value of 131 ng/L for this substance found in the week 8 effluent. [a]Jobling et al. 2003; [b]Arsenault et al. 2004 (NOTE: 20 ug/L was the only level tested); [c]Routledge et al. 1998;

The *in vitro* hER activity levels of 56-106 ng/L measured in the effluent samples also indicated significant reproductive effects potential for the wastewater. This taken together with the E1 [1.4] and E2 [1.1] results from Table 3-1, shows that the effluents arising from this plant in full concentrated form may have the capacity to significantly induce vitellogenin synthesis which may lead to adverse reproductive effects in exposed aquatic organisms. However, considerable dilution in the receiving environment (river delta) of the final effluent occurs and thus, organisms in the environment including Pacific salmon are expected to be exposed to a much lower level of estrogenic compounds and resultant estrogenicity.

3.3.2 Sex specific effects in chinook salmon

The chinook salmon eggs exposed to 100% or 10% TF/SC-primary effluent or 100% TF/SC-secondary effluent from the eyed stage of development onwards experienced 100% mortality shortly after hatching. There was no significant difference in mortality rates between the remaining treatment groups. The average mortality rate in these remaining treatment groups from the eyed stage of development to sampling was 24%.

In all remaining treatment groups, which were sampled at 5 months posthatch, the genetic sex of the fish was found to correspond with the cellular phenotype of the gonads. Fish identified as genetic females presented ovaries containing synchronous oocytes at the perinucleolar stage. Fish identified as genetic males presented testes with spermatogonia and spermatocytes surrounded by sertoli-like cells. No evidence of sex reversal or intersex was apparent in any of the treatment groups. Afonso et al. 2002 found one intersex fish out of 90 fish (44 genetic males) exposed to 10-30% primary effluent and no cases of sex reversal in a previous sampling campaign for the same TF/SC plant sampled here. In this work, in the one surviving treatment group exposed to the more environmentally relevant 1% primary effluent we found no evidence of intersex or sex reversal. This may indicate that in the 1% primary effluent treatment the levels of estrogens are too low to cause intersex or sex reversal in chinook when exposed during the labile period of sexual differentiation. However, the possibility that the sexual differentiation of some sensitive individuals in a population could be impacted by exposure to low levels of primary effluent cannot be excluded at this stage given the very low incidence of intersex noted in previous studies.

In this study, chinook salmon were exposed to 1%, 3%, or 10% secondary effluent during the labile period of sexual differentiation and no cases of intersex or sex reversal were observed. Afonso et al. 2002 exposed chinook salmon to 10%, 30%, and 100% secondary effluent during this labile period and found one intersex fish and two sex reversals in the chinook exposed to 30% secondary effluent (24 genetic males assessed), and one intersex fish in the group exposed to 100% secondary effluent (6 genetic males assessed), and no intersex or sex reversal in the fish exposed to 10% secondary effluent (20 genetic males assessed). Additionally, in Afonso et al. 2002 it was shown that six out of fifteen males exhibited complete sex reversal or intersex when exposed to 1,000 ng/L E2 whereas only 1 intersex out of 10 males resulted in 100 ng/L E2 exposures. In this study, more environmentally relevant concentrations of effluent were tested and, similar to Afonso et al. 2002, we found no evidence of intersex or sex reversal in the fish exposed to 10% secondary effluent (30 genetic males assessed). Additionally, we also found no overt impacts on sexual differentiation in this species following exposures to 3% or 1% secondary effluent (total of 50 genetic males assessed). Secondary effluent concentrations of <10% are more relevant for this TF/SC treatment plant given the high dilution of the effluent following discharge to the Fraser River. This indicates that exposure of chinook salmon to environmentally relevant concentrations of secondary effluent from this TF/SC plant would not be expected to cause intersex or sex reversal in this species, although more subtle effects on reproduction could still be occurring. There are significant interspecies and intraspecies differences in sensitivity to estrogen exposure and further testing of other species likely to be exposed would be required to perform a risk assessment on this aspect.

In this study, the Chinook eggs exposed were not susceptible to intersex or sex reversal at ecologically relevant concentrations of up to 10% final effluent corresponding to estrogenic loads of 7 ng/L E2-equivalents of hER activity. This estimated minimum dilution level in the receiving environment would correspond to environmental estrogen levels of 1 μg/L NP [1.2], 20 ng/L BPA [1.3], 4 ng/L E1 [1.4], and <1 ng/L of E2 [1.1] and E3 [1.5], with sporadic levels of synthetic estrogens EE2 [1.6] and Norgestrel [2.2] based on a 8 week sampling of treated effluents.

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Chapter4- Performance of a tertiary activated sludge municipal wastewater treatment plant in the reduction of estrogenic micro-pollutants

This chapter is a manuscript which has recently been submitted for consideration to *Environmental Science and Technology*. This work represents a detailed mass balance analysis of 40 eEDCs and related compounds over a seven-day sampling period isolating each of the major treatment processes / operations in a full scale BNR plant. The data is fit to a sorption / degradation model previously developed to derive equilibrium and kinetic constants which provide quantitative evidence for problematic removal seen for certain key eEDC compounds typically found in treated municipal wastewater.

4.1 Introduction

Increasing concern regarding very biologically active, and in most cases soluble, substances classed as endocrine disrupting compounds (EDCs) and more recently Pharmaceuticals and Personal Care Products (PPCPs) contained in wastewater effluents has resulted in a multitude of publications over the last 5 to 10 years. Environmental estrogens are a subset of EDCs thought to be responsible for the majority of adverse reproductive effects seen in several fish species in association with municipal wastewater effluents (Hinck et al. 2007; Jobling et al. 1998). The issue of estrogenic EDCs (eEDCs) in wastewater has been recognized by scientists around the world and attention has been given to wastewater treatment as a means to mitigate environmental damage from eEDCs. In Canada, the city of Toronto has already implemented the Water and Wastewater Services Division Sewer Use By-law (By-law No. 855-2002) with Pollution Prevention Planning requirements regulating the levels of specific eEDCs, nonylphenol polyethoxylates (NPEOs) and nonylphenols (NPs), allowed in the sewer system.

The order for general reduction efficiencies of target eEDCs via different types of treatment processes is: membrane biological reactors (MBRs) > activated sludge treatment (AST) with nutrient removal > AST without nutrient removal > trickling filtration (Andersen et al. 2003; Drewes et al. 2005; Joss et al. 2004; Kirk et al. 2002; Shi et al. 2004). A national survey of 28 WWTPs was conducted by Environment Canada's Wastewater Technology Center and National Water Research Institute from 1997-2000 to investigate the occurrence and fate of EDCs in Canadian WWTPs. A strong correlation was found between EDC reduction efficiency and operational process parameters including hydraulic retention time (HRT), sludge age (SRT) and food to micro-organism (F/M) ratio (Anon. 2000). The authors of this study recommend that a better understanding of the EDCs removal mechanisms is required to optimize and predict the removal efficiency of EDCs in WWTPs.

Work completed previously (Fernandez et al. 2007a; Fernandez et al. 2007b) on the levels of various eEDCs in Canadian municipal wastewaters showed that 19-Norethindrone was the most frequently detected and abundant (26–224 ng/L) of all the synthetic estrogens/progesterones in the influent samples. 17α-Ethinylestradiol [1.6] was the more frequently detected synthetic estrogen/progesterone in the effluents occurring at or below 5 ng/L with some sporadic occurrences of up to 178 ng/L. The greatest concentrations of natural steroidal estrogens in municipal effluents were those of estrone (E1) [1.4] >estradiol (E2) [1.1] >estriol (E3) [1.5] which were all <20 ng/L. In some instances, in-plant production of natural estrogens was noted. Nonylphenol (NP) [1.2] and di(2-ethylhexyl) phthalate (DEHP) [2.18] were found in the highest concentrations of the non-steroidal synthetic compounds surveyed in both municipal influent and effluent samples, both occurring at 6–7 μg/L in municipal effluents. Compared to the municipal effluents, pulpmill effluents contained relatively large amounts of the plant sterol stigmasterol [2.9] (4 μg/L), but low amounts of fecal sterols and steroidal estrogens (E2 [1.1] only at 6 ng/L). Overall, the most important substances from a endocrine disrupting potential perspective are, E1 [1.4], E2 [1.1], E3 [1.5], NP [1.2], and any synthetic estrogens present (i.e. 17α-ethinylestradiol [1.6], diethylstilbesterol [4.1]) (Desbrow et al. 1998; Fernandez et al. 2007b; Hewitt and Servos 2001).

State-of-the-art eEDC monitoring tools were used in this work, including, gas chromatography, high resolution mass spectrometry (GC-HRMS), liquid chromatography coupled to triple quadrupole mass spectrometers (LC-MS/MS), and a recombinant yeast assay (RYA) combined with rapid same-day sample preparation techniques. A seven-day multi-point sampling campaign based on flow-proportional 24-hour composites allowed for a detailed and accurate examination of the occurrence, in-plant production and removal rates of the sometimes elusive chemicals which make up the eEDC class in municipal wastewater. Additionally, the data obtained from a municipal WWTP were fit to an existing mechanistic model (Joss et al. 2004) to obtain kinetic and equilibrium constants for the eEDCs of greatest risk to the reproductive health of aquatic organisms.

4.2 Experimental Methods

4.2.1 Sample collection and treatment

In the summer of 2006, several 24-hour flow proportional wastewater samples were collected over seven days (Monday to Sunday) from a tertiary treatment biological nutrient removal (BNR) facility serving an urban population of 750,000. The plant treats an average annual daily flow of 250 MLD which represents 80% of the biological treatment design capacity. Biological treatment is achieved in ten plug-flow nutrient removal bioreactors (C, N, P removal), with a HRT of 6 to 7 hours, and mean SRTs of 7 to 8 days, or 4 to 5 days, in winter and summer, respectively. UV disinfection of clarified effluent is achieved using 4 channels, each equipped with two banks of 90 high-intensity, medium pressure, self-cleaning UV lamps and results in a 99.9% reduction in coliform counts. A 5 MLD sidestream from UV treatment is diverted to membrane modules that pretreat the flow for subsequent industrial re-use. The plant treatment sequence and sampling points are shown in Figure 4-1.

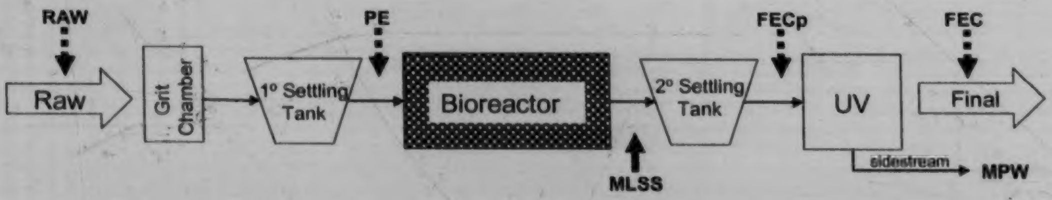

Figure 4-1. Municipal WWTP sequence and sampling points: RAW = raw wastewater. PE = primary effluent; MLSS = mixed liquor suspended solids (daily grab sample from bioreactor#1); FECp = final effluent composite prior to UV; FEC = final effluent composite; MPW = membrane permeate water.

On the day of collection, RAW, PE and MLSS samples were gravity separated from their solids fraction on 16 μm Whatman 150 mm diameter qualitative filter paper. A parallel sample of MLSS was taken for total suspended solids (TSS) determination, heated at 105°C for 4-6 hours. TSS values for PE and RAW solid samples were obtained from the on site plant laboratory for the appropriate days sampled. The RAW and PE 16 μm filter permeate, and FECp and FEC samples were vacuum filtered on Whatman GF/D 47mm disk filtered (2.8 μm) and 2% of total filtered volume in methanol (HPLC grade) was used to rinse each filter and this rinsate was combined with filtrate to include any potential analytes of interest bound to the fine particulate mater in the liquid samples. Samples of PE, RAW and MLSS solids retained on the 16 μm filter paper was loaded into 33-mL stainless steel extraction cells from Dionex, and thoroughly mixed with activated florisil (2 to 3 g for PE and RAW samples and 3 to 4 g for MLSS samples).

4.2.2 Sample Extraction and Preparation

The following extraction techniques were modified from those previously published in Isobe et al. 2003 for liquid wastewater samples and Golet et al. 2002 for the solids, with substantial method development trials used to optimize conditions for the particular suite of analytes studied (see following paragraphs for further details).

Liquid samples: Samples bound for targeted chemical analysis were spiked with 50 µL internal standard mixture (ISM; see Table A4-1 in Appendix VIII) and a replicated sample was extracted similarly without ISM added for yeast assay analysis). Two hundred to five hundred milliliters of each filtered liquid sample was extracted on a 500 mg (6 cc) Waters Oasis® HLB solid phase extraction (SPE) column using Supelco Visiprep® samplers. SPE cartridges were eluted with 7.5 mL aliquots of each of the following solvents: methyl *tert*-butyl ether (MTBE) – fraction 1 (Fr1); diethyl acetate – fraction 2 (Fr2); methanol – fraction 3 (Fr3). Method development trials showed that the elution solvents and order provided optimal signal / noise ratio for the majority of the analytes targeted. These trials included three different elution regimes where the one chosen was adapted from SPE elution scheme #2. Elution scheme #1 was Fr1 = diethyl ether; Fr2 = ethyl acetate; Fr3 = methanol with 5 mM TEA; #2 was Fr1 = diethyl ether; Fr2 = diethyl acetate; Fr3 = 80% methanol; #3 was Fr1 = diethyl ether/10% isopropanol, Fr2 = ethyl acetate; Fr3 = methanol Fernandez et al. 2007c (see Appendix IX for crude peak areas for labeled recovery standards used in method trials to select optimal elution method). No further clean-up was necessary and these fractions were ready for LC-MS/MS and GC-HRMS analysis.

Solid Samples: A Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA) equipped with a solvent controller was used for extraction. Various extraction conditions were tested, and a 20:80 methanol:dichloromethane mixture was found to be optimal. The selected operating conditions were as follows: extraction temperature, 75°C; extraction pressure 13,800 kPa (2000 psi); preheating period, 5 min; static extraction period, 5 min; solvent flush, 50% of the cell volume; nitrogen purge, 60s; and number of extraction cycles, 3 for sewage sludge and sludge-treated soil samples, respectively. Validation of the ASE method was performed previously Fernandez et al. 2007c where duplicate mean BPA-d_4 and Equilin-d_4 recoveries of 106% and 73% were found, respectively.

ASE extracts of the solid samples were split 50:50 volumetrically then one fraction was spiked with 50 μL internal standard (ISM; see Table A4-1 in Appendix VIII) mixture for instrumental analysis and the other was used for yeast assay analysis with no spiking of internal standards (as the compounds would interfere with the assay results). Sparing amounts of aluminum sulfate ($Al_2(SO_4)_3$) were used for drying under a gentle stream of nitrogen and no more than 50 kPa vacuum was applied.

4.2.3 Yeast assay and instrumental analysis

The recombinant yeast assay (RYA) described in Fernandez et al. 2007a was used to detected the presence of human estrogen receptor agonists in the SPE fractions 1-3 for each liquid sample and non-spiked ASE extract for each solid sample. Two complementary instrumental analysis methods LC-MS/MS and GC-HRMS were selected to provide accurate and sensitive detection for the wide range of selected eEDCs for this work (see Table 4-1). Most of the substances chosen for analysis have been found to contribute the majority of estrogenic activity measured in municipal wastewater effluents from the plants surveyed in Fernandez et al. 2007b and in other studies (Desbrow et al. 1998; Nakada et al. 2004). Additionally, conjugate forms of many important wastewater derived eEDCs were selected for analysis. The GC-HRMS method was applied to SPE fraction 1 and forisil cleaned aliquot of the ASE extracts and has been described in detail in Ikonomou et al. 2007 (forisil clean-up details can also be found here) and briefly in Fernandez et al. 2007b (or Chapter 2, section 2.2.2). This technique was employed here to analyze less polar compounds and/or substance which showed poor sensitivity either via APCI or ±ESI LC-MS/MS. LC-MS/MS was used for more polar eEDCs, and particularly the conjugated steroidal estrogens (Isobe et al. 2003) which could not be analyzed by any GC based method due to the extremely low volatility of these substances. SPE fractions 1 and 2 were combined and analyzed for free estrogens and other xenoestrogens (see Table 4-1), whereas, fraction 3 was analyzed for conjugated estrogens (see Table 4-1).

Combined fraction 1 and 2 (i.e. Fr 1/2) was split 80:20 for LC-MS/MS analysis and GC-HRMS, respectively.

Table 4-1. Analytes measured and chemical analysis method used in this work.

Compound	*Description*	*Fraction*	*Analysis*
Estrone-3-sulphate [4.2]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estrone-3-glucuronide [1.19]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estradiol-3-sulphate [4.3]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estradiol-3-glucuronide [1.18]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estriol-3-sulphate [4.4]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estriol-3-glucuronide [4.5]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Estriol-16-glucuronide [4.6]	Conjugated natural estrogen	3	LC-(-ESI)-MS/MS[1]
Ethinylestradiol-3-sulphate [4.7]	Conjugated synthetic estrogen	3	LC-(-ESI)-MS/MS[1]
Ethinylestradiol-3-glucuronide [1.20]	Conjugated synthetic estrogen	3	LC-(-ESI)-MS/MS[1]
17α/β-Estradiol [1.1]	Endogenous female hormone	1/2	LC-(-ESI)-MS/MS[2]
Estrone [1.4]	Endogenous female hormone	1/2	LC-(-ESI)-MS/MS[2]
Estriol [1.5]	Endogenous female hormone	1/2	LC-(-ESI)-MS/MS[2]
16α-hydroxyestrone [4.8]	A major metabolite of estrone	1/2	LC-(-ESI)-MS/MS[2]
17α-Ethinylestradiol [1.6]	Synthetic ovulation inhibitor	1/2	LC-(-ESI)-MS/MS[2]
19-Norethindrone [2.1]	Synthetic ovulation inhibitor	1/2	LC-(+ESI)-MS/MS[2]
(-)-Norgestrel [2.2]	Synthetic ovulation inhibitor	1/2	LC-(+ESI)-MS/MS[2]
Diethylstilbesterol [4.1]	Synthetic hormone	1/2	LC-(-ESI)-MS/MS[2]
Progesterone [4.9]	Natural pregnancy hormone	1/2	LC-(+ESI)-MS/MS[2]
Equilenin [1.7]	Hormone replacement therapy	1/2	LC-(-ESI)-MS/MS[2]
Equilin [2.4]	Hormone replacement therapy	1/2	LC-(-ESI)-MS/MS[2]
α-Zearalanol [2.5]	Veterinary growth promoter	1/2	LC-(-ESI)-MS/MS[2]
17β-Estradiol-3-benzoate [2.6]	Veterinary growth promoter	1/2	LC-(+ESI)-MS/MS[2]
Testosterone [2.7]	Endogenous male androgen	1/2	LC-(-ESI)-MS/MS[2]
β-Sitosterol [1.9]	Major plant derived sterol	1/2	GC-HRMS
Campesterol [2.8]	Major plant derived sterol	1/2	GC-HRMS
Stigmasterol [2.9]	Major plant derived sterol	1/2	GC-HRMS
Stigmastanol [2.10]	Major plant derived sterol	1/2	GC-HRMS
Pinosylvin [1.8]	Stilbene found in *Pinus* species	1/2	GC-HRMS
Cholesterol [1.16]	Animal derived sterol	1/2	GC-HRMS
Coprostanol [1.15]	Cholesterol derivative	1/2	GC-HRMS
Desmosterol [2.11]	Cholesterol derivative	1/2	GC-HRMS
Ergosterol [2.12]	Main sterol produced by fungi	1/2	GC-HRMS
6- ketocholestanol [2.13]	Cholesterol oxidation products	1/2	GC-HRMS
7-ketocholesterol [2.14]	Cholesterol oxidation products	1/2	GC-HRMS
Coprostan-3-one [2.15]	Fecal neutral sterol	1/2	GC-HRMS
Fucosterol [2.16]	Sterol found in seaweed	1/2	GC-HRMS
Totarol [2.17]	Antibacterial diterpenoid	1/2	GC-HRMS
Di-(2-ethylhexyl)phthalate [2.18]	Ubiquitous plasticizer / phthalate	1/2	GC-HRMS
Nonylphenol [1.2]	EDC from non-ionic surfactants	1/2	GC-HRMS
Bisphenol A [1.3]	Plasticizer / intermediate in PVC	1/2	LC-(-ESI)-MS/MS[2]

[1]LC method 1; [2]LC method 2 (see Appendix X for details).

The GC-HRMS system consisted of a Hewlett Packard 5890 Series II Gas Ghromatograph coupled to a VG AutoSpec magnetic sector mass spectrometer (Micromass UK Ltd., Manchester, UK). For GC-HRMS analysis, sample derivatization was followed as described in Fernandez et al. 2007b (or Chapter 2 section 2.2.2.) and in further detail in Ikonomou et al. 2007. The LC-MS/MS was the API 5000 triple quadruple equipped with a Summit Dionex HPLC system and positive and negative ESI was used (See Table 4-1 for details on each compound). Additionally, two different liquid chromatographic methods were necessary to capture all the compounds analyzed by LC-MS/MS in Table 4-1 as specified in the footnotes of this table (see Appendix X for details of each chromatographic method). Final extracts were taken to incipient dryness under nitrogen and re-suspended in the solvent mixture corresponding to the initial mobile phase composition in each LC analysis. Finally, 10 µL of a 9 ng/µL 17β-estradiol-17-acetate performance standard added prior to LC-MS/MS analysis. LC method#1 (i.e. conjugated estrogens) used gradient elution with acetonitrile and water with a modifier of 10mM formic acid D'Ascenzo et al. 2003, an Xterra® 5µm C_{18} 30mm x 4.6mm (Waters) column, and a 1000 µL/min flow rate with a 3/4 split to waste. LC method#2 used gradient elution with methanol and water with 0.01% NH_4OH modifier Fernandez et al. 2007c a Gemini® 3µm C_{18} 100mm x 2mm (Phenomenex) HPLC column was used at a flow rate of 200 µL/min and a column oven temperature of 40°C. These conditions were determined to provide optimal chromatographic separation and facilitate the generation of ions for the desired analytes.

Table A4-2 in Appendix VIII provides the LC-MS/MS ion transitions monitored for each compound. Two to three ions were used for confirmation of each compound, and the most intense ion was then used for quantitation as specified in the footnotes of this table. The ionization and product ion formation of each compounds was individually optimized using automated optimization available with the API 5000 by systematically varying the declustering potential (i.e. analogous to standard cone voltage) and collision energy (in the Q2), to obtain maximum product ion formation (See Appendix XI for illustrations of the various components of a typical MS/MS unit). Additionally, the analyst optimized the interface temperature and nebulizer / curtain gas flow rates to maximize molecular ion formations for most of the compounds analyzed (API 5000 interface is confidential and no diagram could be provided). With the optimized instrument method very low detection limits were obtained ranging from 0.02 to 3 ng/L for all the substances measured. Internal standard calibration was used for all compounds except for LC-MS/MS runs of bisphenol A, where internal standards calibration showed poor results due to poor IS response; external calibration was used in these instances. BPA recovery was recovery corrected to 66% which was the mean recovery of two triplicate spike trials with PE and FEC. Eight point calibration from 1 ng to 1000 ng (1 mL final volume; exact final volumes were used of external calibration standards and samples) or maximum amount of compound just prior to saturating the detector was typically used, yielding a quadratic calibration curve with $R^2 > 0.99$. Blanks, duplicates and spikes were included as QC samples for each batch of ten samples. Recovery accuracy and method precision was based on triplicate matrix blank-subtracted spikes of both PE and FEC samples collected on the final day of sampling. Internal standard recoveries were normally between 60-120% as a QA/QC criterion. E1 and E2 were further recovery-corrected based on matrix blank-subtracted spike recoveries due to the high potency and extreme importance of an accurate absolute amount determination of these compounds to the hormonal activity of wastewater.

4.2.4 Mass Balance Calculations for all eEDCs

Percent reduction (PR) observed in the eEDCs measured throughout the treatment train was calculated as:

$$PR = \left[\left(\sum m_{s(in)} + \sum m_{d(in)} - \sum m_{s(out)} - \sum m_{d(out)}\right) \div \left(\sum m_{s(inlet)} + \sum m_{d(inlet)}\right)\right] \times 100\% \qquad (4\text{-}1)$$

Where "$\sum$m" signifies the total mass of substance passing through the treatment works over the seven-day sampling period. The "in" and "out" subscripts relate to the influent and effluent, respectively of each treatment stage isolated in the sampling. Subscript "s" signifies a chemical species associated with the solid fraction and "d" signifies a chemical dissolved species. The systematic error due to the hydraulic lags between the four sampling points from RAW to FEC, was minimized by basing the percent reduction calculation on a seven-day period.

The flux of eEDCs associated with adsorption to new microbial growth during secondary treatment was based on sludge production rates (gSS/m^3 wastewater treated) calculated as a mean between observed (4-2) and predicted (4-3) rates for the plant sampled:

$$\overline{SS}_{production(obs)} = \left[\sum \left(V \times MLSS_{i+1} - V \times MLSS_i + Q_{waste} \times MLSS_i\right) \times 9 \div Q_{overall} - TSS_{inf} + TSS_{eff}\right] \div 7days \quad (4\text{-}2)$$

Where V = volume of each of the 9 (one of the 10 bioreactors was down for maintenance during the sampling period) bioreactors run in parallel; MLSS = mix liquor suspended solids concentration on day *i* and day *i*+1; Q_{waste} = daily waste activated sludge flow; $Q_{overall}$ = daily wastewater flow; TSS_{inf} = suspended solids concentration for bioreactor influent (i.e. primary effluent); TSS_{eff} = suspended solids concentration of bioreactor effluent (i.e. FECp).

$$\overline{SS}_{production(predicted)} = \left[Y_{obs} \times \left(S_0 - S\right) \times 1.15 + Y_{NOx} \times NOx\right] \div 0.85 \qquad (4\text{-}3)$$

Equation (4-3) is based on formulae in Tchobanoglous et al. 2003: Y = g VSS/ g substrate for biomass production by aerobic heterotrophs (Y_{obs}), and nitrifying bacteria (Y_{NOx}); S_0 = BOD of influent; S = BOD of effluent (S); NO_x = amount of NH_4-N converted to nitrate (estimated as 25 mg/L for this plant based on influent and effluent TKN and NH_4 data for sampling months); 1.15 factor to account for 15% increase sludge production due to PAOs (Joss et al. 2005); 0.85 factor is assuming 80-90% of TSSs are VSSs (Tchobanoglous et al. 2003).

The best estimate of sludge production was 83 g SS / m^3 obtained by taking the mean of the observed (104 g SS / m^3) and predicted (62 g SS / m^3) values. The observed value was limited by the fact that MLSS was only measured accurately for bioreactor 1 (and applied to all 8 other online bioreactors), and the predicted value may suffer from the inaccuracies associated with obtaining biomass production rates from typical values observed for conventional activated sludge plants published in Tchobanoglous et al. 2003, knowing that subtle differences in biodiversity of floc and wastewater composition can affect this rate significantly.

Micropollutant removal via adsorption to new sludge (RVS) during secondary treatment was calculated using equation (4-4):

$$RVS_x = \overline{SS}_{production} \times Q \times C_{s,floc} \qquad (4\text{-}4)$$

$C_{s,floc}$ = concentration of substance sorbed to sludge (e.g. ng / gram of MLSS).

4.2.5 Modeling and Error Estimation for key eEDCs

The modeling approach taken in this work was adapted from the previous work (Joss et al. 2004; Joss et al. 2005). It was found that the natural female estrogens E1 and E2 were the greatest sources of estrogenic activity in the sampled wastewater and thus, only data from the three female endogenous estrogens were used in the modeling and Monte Carlo simulation as this analysis was computationally intense. Monte Carlo simulation with 5000 runs was used to determine estimates of error for the degradation and deconjugation rates as well as solid-liquid partitioning coefficient; the input variables varied randomly according to their distributions (see Appendix XII for an example of a Monte Carlo run for solid-liquid sorption coefficient, K_D error determination for the compound E1 as in equation 4-6). Error estimates for eEDC concentrations were obtained from triplicate spike data and converted to multiplicative standard deviation by rising to the power of ten as concentration values are log-normally distributed (see Appendix XIV for %Log SD and multiplicative standard deviation determined for each chemical measured used in this work). Error estimates for Mean $SS_{production}$ and Q which were used to determine the solid and liquid mass fluxes for eEDCs was */1.2 (multiplicative SD) and ±10% (normal %SD) respectively as recommended by plant personnel and in the literature (Joss et al. 2005).

4.3 Results and Discussion

4.3.1 Removal efficiency for eEDCs

The percent removal of all detected compounds (from all the substances measured, see Table 4-1) measured based on inlet mass loading (i.e. g/day) for each treatment stage of the WWTP is shown in Figure 4-2 (see Appendix XIII for all raw wastewater quality, operational and chemical contaminants data obtained and used in this work). Based on the data presented in Figure 4-2, both primary and secondary treatment stages play significant roles in the reduction of several eEDCs. In some cases nearly 100% removal was seen for the natural steroids progesterone, testosterone and estriol; plant sterols (i.e. β-sitosterol); and to a lesser extent bisphenol A. Less than satisfactory reduction was seen for estradiol, estrone and their parent glucuronide and sulfate conjugates, and for nonylphenol. In plant production of RYA measured E2-equivalents was seen at every treatment stage including UV. In addition, estrone showed in-plant production during both primary and secondary treatment stages. However, UV treatment had a reducing effect on the residual estrone in the effluent. This in-plant production of E1 during primary treatment is likely due to deconjugation of conjugated E1-glucuronide which is know to occur even during transit in the sewers in part due to exctracellular β-glucuronidase from *E.coli* bacteria, a known constituent in raw wastewater (D'Ascenzo et al. 2003).

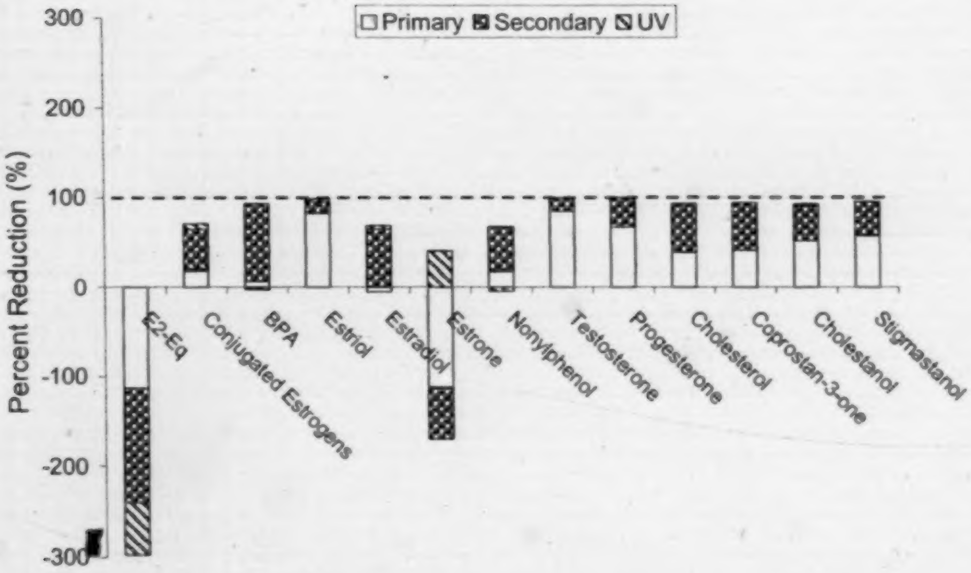

Figure 4-2. Percent removal of several eEDCs detected above detection limits based on inlet loading rate (g/day) over seven-day sampling period by treatment stage.

As no significant hydraulic lag was present in the UV treatment stage, mean daily percent removal for estrone via UV could be accurately calculated with a value of 45±36% (95% confidence interval based on 6 available daily pairs of measurements). Thus, destruction of estrone by UV was found to be considerable. UV disinfection, as performed at this plant shows promise in reducing the levels of E1 ultimately discharged into the environment.

4.3.2 Removal Processes for eEDCs

The focus of the next part of this work was to determine the predominant mechanisms of formation and removal of the substances which contributed most to the estrogenic levels of the wastewater. As we calculated the solids removal via sludge in equation (4-4) and total removal in equation (4-1), the difference would be equal to the amount removed via biotransformation / biodegradation and any air stripping. None of the analytes monitored where good candidates for air stripping based on Henry's law constant (HLC), all $<10^{-4}$ atm m^3 / mol aside from nonylphenol (NP) [1.2] which has a HLC of 11 atm m^3 / mol (Birkett and Lester 2003).

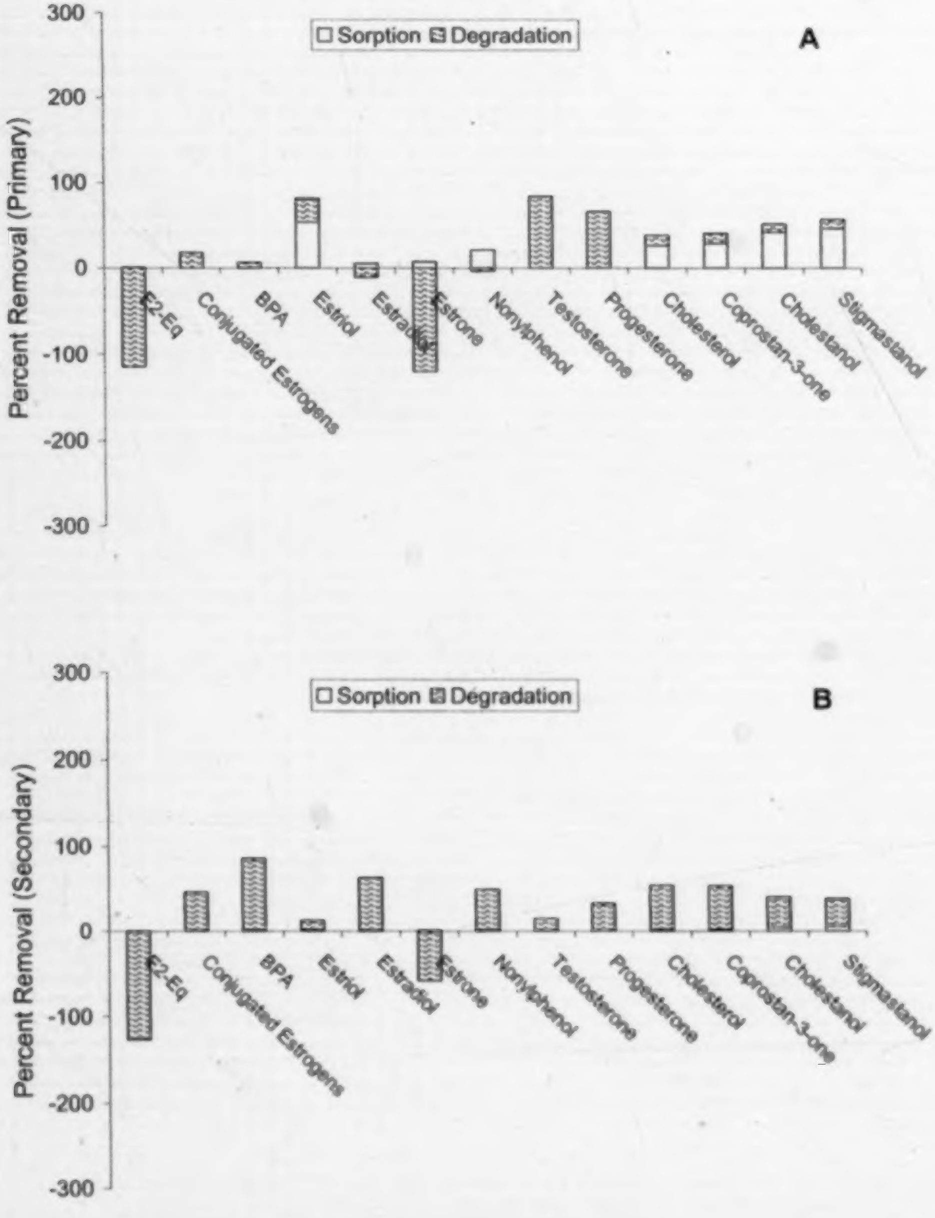

Figure 4-3. Percent removal of several eEDCs detected above limits of detection based on inlet loading rate (g/day) over seven-day sampling period based on amount removed in sludge versus "degradation" for (A) primary treatment stage and (B) secondary treatment stage.

For primary treatment, it was assumed that no new sludge was produced and thus, observed removal was only a function of eEDC adsorbed to solids and observed solids removal (TSS_{RAW} - $TSS_{Primary}$ as seen in Table A4-3 Appendix XIII). Figure 4-3, shows the relative importance of sorption to sludge versus "degradation" (in quotation, since as discussed, all compounds show unfavorable HLC for air stripping, however the atmosphere above the wastewater was not monitored for eEDCs). Adsorption to sludge may be an important removal mechanism for some of the eEDCs during primary treatment (as many substances may have been associated with the solids from their initial entry into the sewers), however, sorption to sludge is minimal during secondary treatment, and only slightly significant for the most hydrophobic eEDCs measured, plant and animal sterols (i.e. stigmastanol [2.10] and cholesterol [1.16], coprostan-3-one [2.15], coprostanol [1.15]).

Although not the focus of this work, Figure 4-3A highlights the importance of primary sludge as a potential route for potent eEDCs NP and estriol into the environment. It remains to be determined how effective anaerobic sludge digestion may be in reducing these substances in the processed sludge. Figure 4-3B, shows that most of the removal of eEDCs in secondary treatment (where greater process control may lend itself to target optimization) is via "degradation". This figure also shows that in-plant formation of E1 and RYA measured activity may be problematic as this is the final stage of biological treatment prior to release of the effluent to natural waters.

4.3.3 Removal of Important eEDCs: E1 and E2

It was determined that E1 and E2 were significant contributors to the RYA measured E2-equivalents based on significant linear regression between the dissolved flux of these measurements throughout the treatment works as seen in Figure 4-4 as well as from data presented in previous work (Fernandez et al. 2007b and Fernandez et al. 2007a or Chapters 2 and 3). These substances are also known to be some of the most estrogenically potent EDCs identified in municipal wastewater effluents with estrogenic equivalence factors of 0.1 for E1 and 1 for E2 (Coldham et al. 1997). A better understanding of the natural steroidal estrogenic substances in secondary treatment was sought as optimization of secondary treatment has been suggested as the most cost-effective and environmentally sustainable solution for mitigating the risks of eEDCs to aquatic life and human health (Johnson and Sumpter 2001; Jones et al. 2007).

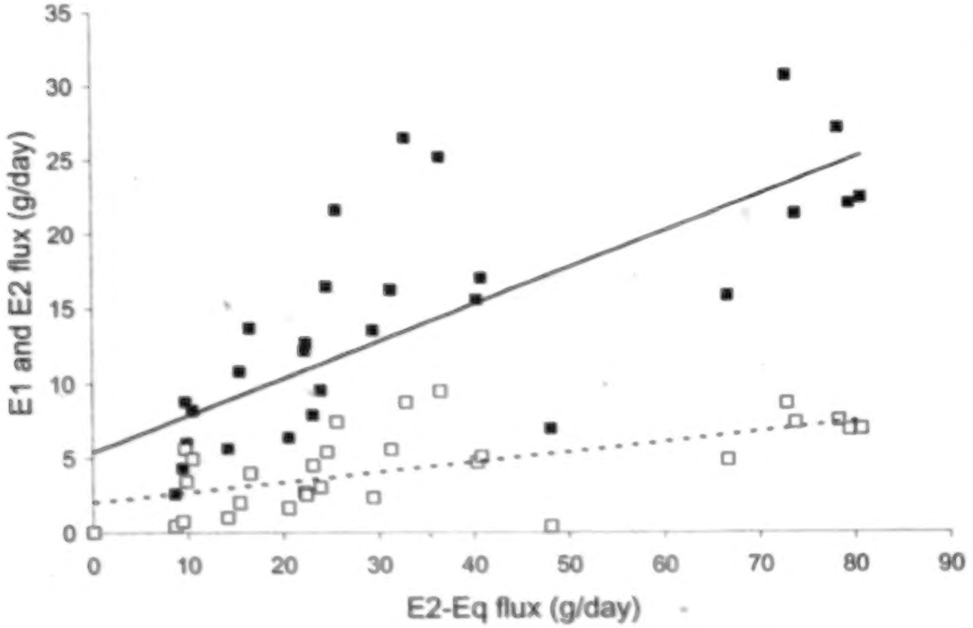

Figure 4-4. Correlation between E1 (solid squares), E2 (open squares) flux and RYA E2-equivalents. $r_{E1} = 0.76$ (solid line); $r_{E2} = 0.62$ (hatched line); n = 31, significant at 95% confidence.

Figure 4-5 shows the flux of the three natural steroidal estrogens during secondary treatment, based on the mechanistic models proposed in Joss et al. 2004 for free and conjugated natural estrogens. Figure 4-5, shows that deconjugation of glucuronic acid and sulphate conjugates significantly contributed to the concentration of natural estrogens in the bioreactor. Additionally, adsorption was insignificant in the removal of estrogens compared with degradation. Both E2 and E3 were reduced by two thirds, whereas E1 concentration was increased in the bioreactor effluent. Clearly, deconjugation played an important role in this increase, however, the oxidation of E2 to E1, will have further contributed to this increase.

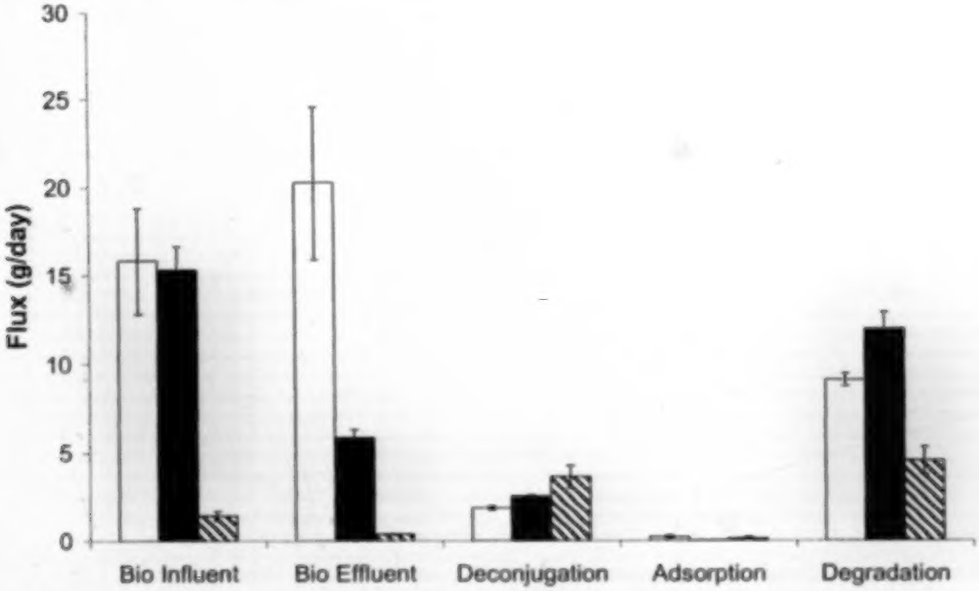

Figure 4-5. Flux of natural steroidal estrogens E1 (open bars), E2 (solid bars), E3 (stripes), during secondary treatment with error estimates (±1 standard deviation) estimated using Monte Carlo simulation.

Pseudo-first order reaction rate constants (k_{bio}) may be related to the volatile suspended solids concentration (VSS), concentration of substance dissolved in the bulk liquid (C_w) and rate of reaction (r) as in Joss et al. 2004 via:

$$r = -k_{bio} \times VSS \times C_w \qquad (4\text{-}5)$$

In Figure 4-5 we have calculated the daily rate of degradation and deconjugation for each of the natural estrogens which may be substituted in equation (4-5) together with the VSS and estimate of C_w to obtain the pseudo-first order reaction rate constants (k_{bio}). The following assumptions were made in this work:

1. All reactants and products were in steady state in the full-scale system.

2. MLVSS concentration was 0.8 x MLSS which was constant in the bioreactors, and only VSS is capable of biodegradation in the system.
3. Effective concentration of reactants was equivalent to the mean of influent and effluent concentrations in the completely mixed bioreactors.
4. It was assumed that all E2 degradation was equal to E1 formation due to the well known E2 → E1 oxidation.
5. For E1, E2 and E3 glucuronide and sulphate conjugates no adsorption to sludge occurred (polarity of these conjugates is much greater than any compound which exhibits significant sludge adsorption)

The calculation of kinetic constants in this work allowed a quantitative assessment of the pathway specific removal intensity for the most important eEDCs (i.e. aquatic sexual disruptors) at a full scale BNR plant.

Sorption rates (k_{sor}) could not be determined as a sampling frequency of several times per day would have been required. This is because in practice, sorption rates for micropollutants occur quickly relative to a HRT (several hours) and new sludge production (200-400 g SS / m^3 wastewater) Ternes and Joss 2006, and thus, any new sludge which is produced during one HRT cycle is rapidly saturated with pollutants based on their solid-liquid partitioning coefficient (K_D). Thus, the solid-liquid partitioning coefficient K_D is a more useful parameter than the sorption rate constant in predicting the amount of substance that will adsorb to sludge assuming that the relatively fast kinetics allow equilibrium conditions to prevail. The solid-liquid sorption coefficient can be calculated for a particular substance in secondary and primary sludge based on K_D as in Joss et al. 2004.

$$K_D = \frac{C_{S,floc}}{C_w} \qquad (4\text{-}6)$$

Where $C_{s,floc}$ is the concentration of estrogen in the sludge floc (ng/gSS); C_w is the concentration of estrogen dissolved in the water column (ng/L). For primary sludge, the amount sorbed to the PE solids fraction was used as $C_{s,floc}$.

Based on the model equations (4-5) and (4-6) together with observed flux rates in Figure 4-4, kinetic and equilibrium coefficients were derived (see Table 4-2) with error estimates in order to provide values which would be intercomparable to batch experiments, pilot plants and other full-scale facilities. This universal comparisons basis may be useful in providing insights into which factors contribute to an efficient removal of these undesirable substances through secondary treatment process of municipal wastewaters.

Table 4-2. Pseudo-first order reaction rate constants (k) and solid-liquid partitioning (K_D) coefficients.

Compound	*k_{biol1} (deconjugation)* *L / gSS*d*	*k_{biol2} (degradation)* *L / gSS*d*	*K_D (primary / secondary)* *L / gSS*
E1	1.6±0.1	0.77±0.03	0.35±0.08 / 0.13±0.03
E2	2.4±0.1	3.5±0.3	0.17±0.01 / 0.07±0.01

Note: mean sampling temperature of 15°C; error is ±1 standard deviation from Monte Carlo simulation

The most significant observation made from examining the values tabulated in Table 4-2 is the relative rates of deconjugation versus degradation, are greater for E1 than for E2. This provides a direct mechanistic explanation as to why E1 was found to accumulate within the treatment works (i.e. since rates of deconjugation proceeded faster than that of degradation making the latter a bottleneck in the elimination of hormonal activity), whereas other potent eEDCs such as E2 did not. From batch experiments performed with diluted sludge from an activated sludge treatment works Joss et al. 2004; Ternes et al. 1999, the rates of degradation of E1 and E2 were more than one order of magnitude greater than the deconjugation rate, suggesting that in the full scale situation the kinetics are not favorable for the removal of E1 compared with batch scenarios. Additionally, the trend in the solids liquid partitioning coefficients, E1>E2, and primary >

secondary for both E1 and E2, agrees well with the literature which reports on these values for eEDCs (Ternes and Joss 2006). This means that E1 has the greatest potential for adsorption to primary sludge, however this K_D is still quite low considering a $K_D < 0.3$ was suggested by Ternes and Joss 2006 as the cut-off for significant adsorption to sludge even at very high sludge production rates. Thus, as shown in Figure 4-4, sorption to sludge (particularly secondary sludge) is not a significant removal mechanism for the most potent eEDCs in municipal wastewater treatment. However, E1 reduction in further processing of primary sludge should be further examined in order to determine any potential risks to human health and the environment via the sludge route for this contaminant.

Several considerations for comparing the results shown in Table 4-2 to different experiments and biological flocs (i.e. sludge) exist. First of all, the batch work cited here was done under aerobic conditions whereas the bioreactors at the plant sampled are 75% aerated by volume, and 25% either anaerobic or anoxic. Additionally, the proportion of active biomass in the sludge along with the biodiversity of the floc will impact the rates of reaction greatly, as will the availability of co-substrates in wastewater. Finally, it still remains to be determined whether degradation of EDCs occurs via extracellular enzymes excreted by microorganisms in the floc, or whether degradation occurs during adsorption on the floc particles or absorption in to the floc particles.

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Chapter5- Seasonal variability in the reduction in estrogenic endocrine disrupting chemicals at a municipal WWTP

This work was recently submitted to *Water Research* for consideration for publication. One of the large gaps in municipal wastewater derived eEDC research is providing a seasonal context to the occurrence and removal performance of these substances. Preliminary seasonal variability effects detected in preliminary work by this author at the aerated lagoon (site E) provided the main impetus for performing the work in this chapter (see section 2.3.3 and Appendix XVI for details of these preliminary results). This chapter reports on a long term monitoring study which was designed to study the associations (if any) between ambient conditions, wastewater treatment operation and water quality with the removal of eEDCs or associated estrogenicity.

5.1 Introduction

Increasing concern regarding very biologically active substances, classed as endocrine disrupting compounds (EDCs) and more recently pharmaceuticals and personal care products (PPCPs), contained in wastewater effluents has resulted in a multitude of publications over the last 5 to 10 years. Environmental estrogens are a subset of EDCs thought to be responsible for the majority of adverse reproductive effects observed in several fish species exposed to municipal wastewater effluents (Hinck et al. 2007; Jobling et al. 1998). The issue of estrogenic EDCs (eEDCs) in wastewater has been recognized by scientists and engineers around the world and attention has been given to wastewater treatment as a means of mitigating current and future environmental damage from eEDCs. The city of Toronto, Canada, has implemented the Water and Wastewater Services Division Sewer use By-law (By-law No. 855-2002) that regulates the allowable levels of the eEDCs, nonylphenol polyethoxylates (NPEOs) and nonylphenols (NPs), allowed in the sewer system.

Recent studies have been conducted to attempt to understand and optimize EDC reduction within activated sludge treatment facilities. Drewes et al. 2005 reported a significant correlation ($R^2 = 0.76$) between influent BOD_5 loading and influent E2-equivalents loading in seven full-scale water reclamation facilities. Other researchers have shown that there is a positive correlation between the removal of eEDCs and HRT and also SRT in full-scale facilities (Holbrook et al. 2002; Johnson et al. 2005). The greater age of sludge associated with higher recycling rates increases the concentration of slower growing, perhaps specialist organisms which degrade estrogens (Joss et al. 2004). Furthermore, an increase in HRT increases the contact time between the dissolved estrogens and mixed liquor suspended solids (MLSS) and thus, increases the time available for adsorption and biodegradation to occur. However, little work has been done to study these relationships in colder climates. It has been very well documented that biological growth and enzymatic reaction rates may increase with ambient temperature conditions. Furthermore, no long-term study examining the effect of operational, wastewater quality and seasonal variables on the removal of eEDCs from municipal WWTPs has been reported in the literature.

Several bio-analytical methods have been developed as surrogate measures of environmental estrogens or of net estrogenic potency of a sample. Enzyme linked immuosorbent assays (ELISAs) (Estevez-Alberola and Marco 2004), radioimmunoassays (RIAs) (Snyder et al. 1999), transcriptional assays, including both reporter gene assays and cell proliferation assays (Routledge and Sumpter 1996; Gaido et al. 1997; Garcia-Reyero et al. 2001; Soto et al. 1995; Legler et al. 1999), have been developed to assess eEDCs in environmental samples. Several recombinant strains of *Saccharomyces cerevisiae* (RYAs) have been developed which incorporate the human estrogen receptor (hER) gene in the main chromosome of the yeast, in addition to expression plasmids carrying the report gene lac-Z encoding for the enzyme β-galactosidase (Routledge and Sumpter 1996). Upon activation of the hER receptor, this enzyme is secreted into the medium and may be assayed using a chromogenic substrate. Advantages of using yeast for eEDC detection include ease of manipulation, rapid attainment of stable transformants, ability to process large samples and a limited metabolic capacity compared with more elaborate bioassay systems (Gaido et al. 1997). For targeted or mass conservative measures of eEDCs, many liquid chromatography - mass spectrometric (LC-MS) based methods have been developed for wastewater and receiving waters (Reviewed in Richardson 2004). However, for a large number of samples with limited sample amount available, bioanalytical methods may be the only option, considering the relatively long sample work-up procedure for LC-MS/MS analysis versus a bio-analytical method.

The goal of the present investigation was to apply a well established and widely used RYA to assess the reduction efficiency of a full-scale BNR plant for this bio-analytical parameter. Complementary instrumental analysis for targeted eEDCs in select samples was also performed. These temporal data are combined with operational, wastewater quality, and climate data to determine which of these variables may be related to the levels and reduction of RYA E2-equivalents, intended as a surrogate measure of important eEDCs for the plant studied.

5.2 Experimental Methods

5.2.1 Sample collection and analysis

The plant chosen for sampling was a tertiary treatment facility serving a predominantly urban population of 750,000 that receives an average annual daily flow of 250 MLD (currently at 80% of its average secondary treatment design capacity). Ten plug-flow nutrient removal bioreactors (C, N, P removal), with a HRT of 6 to 7 hours, and mean SRTs of 7 to 8 days, or 4 to 5 days, in winter and summer, respectively. UV disinfection of clarified effluent is achieved using 4 channels, each equipped with two banks of 90 high-intensity, medium pressure, self-cleaning UV lamps and results in a 99.9% reduction in coliform counts.

Twenty-four hour flow proportional composite samples each from primary effluent (PE) and final effluent (FEC) collected daily were sub-sampled approximately every Monday and Thursday from September to December, 2006 (see Figure 5-1). Each sample was filter sterilized using 0.2 μm Puradisc® cartridge filters (Whatman, Middlesex, UK) and stored in sterile glass vials at -20°C for collective αhER recombinant yeast assay (RYA) analysis.

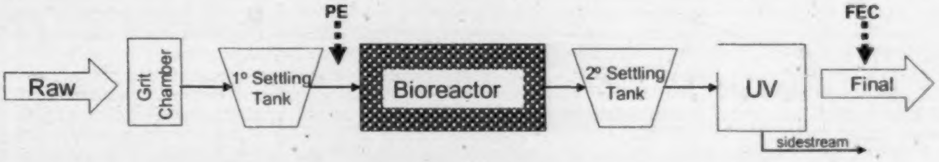

Figure 5-1. Flow diagram for WWTP sampled in this work, showing two sets of sample which were taken. PE = primary effluent composite; FEC = final effluent composite.

RYA was carried out in 96-well plates as described in Fernandez et al. 2007b (or Chapter 2 section 2.2.3). Samples were run in 3 serial dilutions of 1:4, 1:12 and 1:24, along with a positive control (0.02 ng E2 per well) for toxicity at the 1:12 dilution level. This was the optimal layout to analyze these samples for estrogenic activity, as both true dose response and toxicity effects could be evaluated in every sample. Concentration in E2-equivalents (ng/L wastewater) was then calculated from a simultaneously run 12 point calibration curve ($n=4$; $r^2 < 0.998$) of E2 from 10^{-4} to 10^{-10} g/L (see Fernandez et al. 2007b and Appendix III for more details). Procedural blank samples were prepared from distilled water run identically to the wastewater samples, such that the blank values could be subtracted from the samples to obtain a final value. E2-equivalents were calculated from the 1:12 dilution unless this dilution did not show a response, in which case the 1:4 dilution value was used. For samples which responded greatly, the 1:24 value was used only if the 1:12 was out of range.

Four 10 mL aliquots (previously analyzed for RYA activity) were thawed and combined for each warm and cold temperature time period. The 30-40 mL composite was SPE extracted, worked-up and analyzed for free and conjugated estrogens via LC-MS/MS as described in detail in Fernandez et al. 2007c (or Chapter 4 section 4.2.3). Each composite was analyzed for the free estrogens: 17α/β-Estradiol [1.1], Estrone [1.4], Estriol [1.5], 16α-hydroxyestrone [4.9], 17α-ethinylestradiol [1.6], 19-norethindrone [2.1], (-)-norgestrel [2.2], diethylstilbesterol [4.1], progesterone [4.10], equilenin [1.7], equilin [2.4], α-zearalanol [2.5], 17β-estradiol-3-benzoate [2.6], testosterone [2.7]; and conjugated estrogens: estrone-3-sulphate [4.2], estrone-3-glucuronide [4.3], estradiol-3-sulphate [4.4], estradiol-3-glucuronide [1.18], estriol-3-sulphate [4.5], estriol-3-glucuronide [4.6], estriol-16-glucuronide [4.7], ethinylestradiol-3-sulphate [4.8], ethinylestradiol-3-glucuronide [4.8]. Internal standard (IS) calibration was used with 3,4-$^{13}C_2$-estradiol for the free estrogens and estrone-2,4,16-d_4-3-Sulfate for conjugates. IS recoveries were normally between 60-120% as a QA/QC criterion and all values were blank subtracted.

5.2.2 Data acquisition and processing

Pertinent data that are collected for regulatory or process purposes were provided for the sampling period by plant personnel. These pertinent data include: Daily air and effluent temperature, total rain fall, wastewater flow rates, influent/effluent biochemical oxygen demand (BOD), influent/effluent total suspended solids (TSS), ammonia, and solids retention time (SRT) (see Appendix XV for a complete listing of all data available during sampling period). Meterological data for the sampling period were obtained from Environment Canada (http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html). Data processing and analysis including calculation of Pearson's correlation coefficient (r), linear regression, and Durbin-Watson's *d* was performed using Statistica 7.1 (Stats Soft, Inc, Tulsa, OK).

5.3 Results and Discussion

5.3.1 Temporal distribution of ambient/operational/regulatory parameters

The RYA activity detected in the PE and FEC samples each Monday and Thursday, along with daily mean ambient temperature, effluent temperature and several other wastewater quality and operational parameters measured from September 11th to December 31st, 2006 are available in Appendix XV. As seen in Figure 5-2, there was significant variability present throughout the sampling period in flow (which is inversely proportional to HRT) and SRT parameters which may have some impact on the treatment efficiency of WWTPs for several organic micropollutants found in domestic wastewater streams (Holbrook et al. 2002; Johnson et al. 2005; Tchobanoglous et al. 2003).

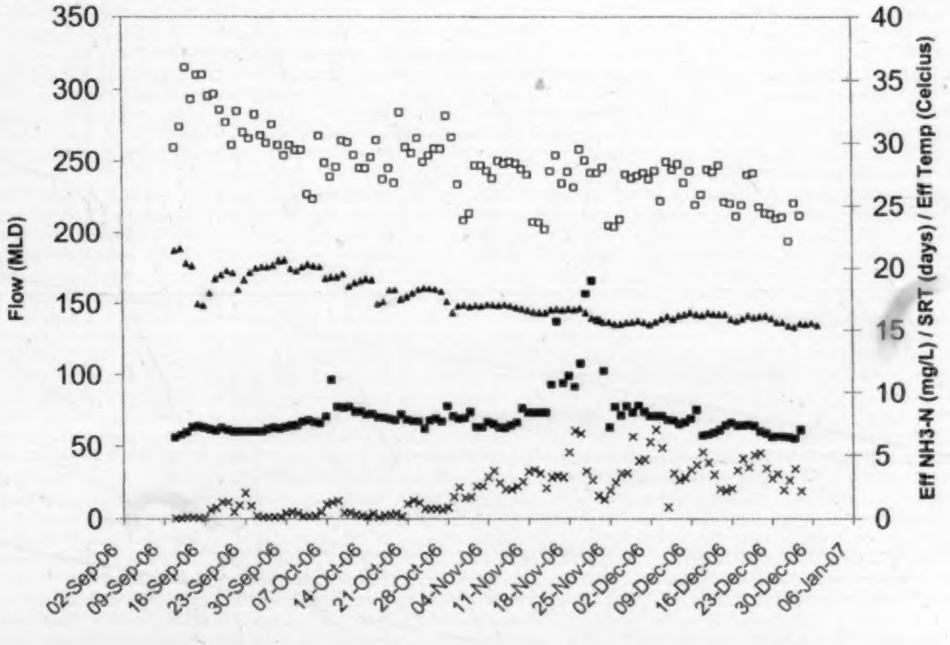

Figure 5-2. Seasonal variation in wastewater flow (open squares), temperature (solid triangles), residual ammonia concentration (crosses), and mean solids retention times of all 10 bioreactors (solid squares).

In addition, the residual ammonia nitrogen (NH_3-N) concentration was plotted as an indicator of the efficiency of pollutant removal (influent ammonia nitrogen levels were relatively constant throughout the sampling period averaging a concentration of 30 mg/L and ranging from 20 to 45 mg/L and were only available for a few of the sampling days). An ammonia nitrogen spike between November 18th and 25th was reduced by plant operators strategically increasing SRT such that nitrifying bacteria, which are relatively slow growing particularly in lower temperatures, had the opportunity to repopulate the bioreactors to achieve efficient ammonia removal. This would be a normal response to deal with ammonia issues in a BNR municipal WWTP, thus, this provided an opportunity to observe any effect of this operational adjustment on the reduction efficiency of estrogenic substances measured in this work. Mean effluent temperature was also considered in this work, as many physical and biologically mediated degradation rates of compounds are temperature dependent (Tchobanoglous et al. 2003). The mean daily ambient air temperature varied by as much as 43°C during the sampling period (see data tabulated under the ambient conditions section of Appendix XV). However, during the sampling period, the effluent wastewater temperature varied by only 7°C, as seen in Figure 5-2. Figure 5-2 also shows the characteristic decrease in wastewater flow rate as temperatures decline.

5.3.2 Temporal distribution of RYA measured estrogenicity

RYA (αhER) was used to measure the activity of each wastewater bioreactor influent and effluent sample collected semi-weekly from September to December, 2006 (see Figure 5-3). Most of the wastewater samples collected during this period show a considerable amount of RYA activity. Previously published work illustrated a statistically significant positive correlation between the flux (g/day) of RYA measured activity and important female endogenous estrogens E1 and E2 measured via LC-MS/MS (Fernandez et al. 2007c). Thus, although RYA is not a mass conservative measurement, due to the lack of compound specificity and likely presences of competing estrogenic and anti-estrogenic substances, it can be taken as a reliable measure of the relative levels of E1 and E2 at this plant. These two compounds were identified as the most problematic eEDCs in municipal wastewater effluents in previous work by this author (Fernandez et al. 2007a;

Fernandez et al. 2007b; Fernandez et al. 2007c or Chapters 2, 3 and 4) and other researchers (Desbrow et al. 1998).

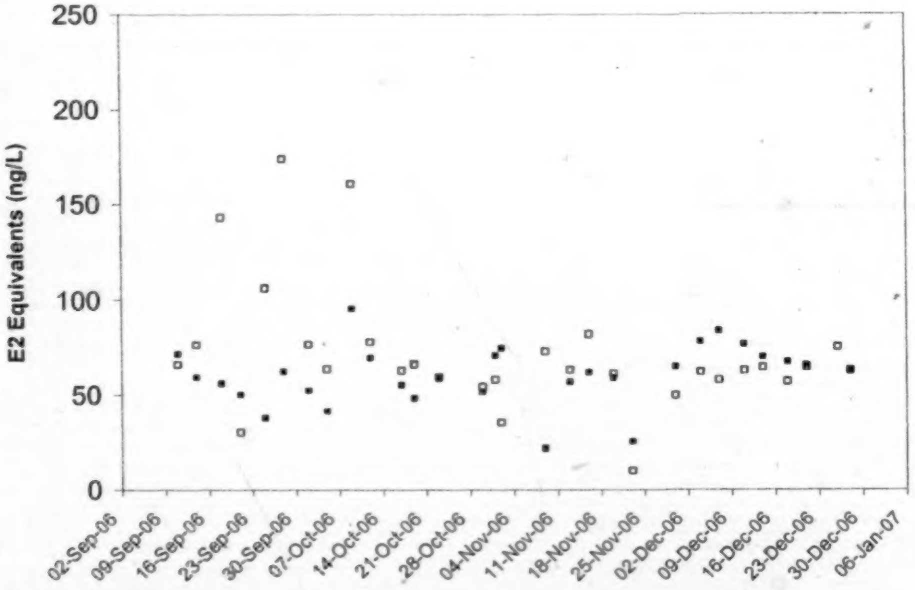

Figure 5-3. RYA measured activity on 0.2 μm filtered whole effluent samples from bioreactor influent (PE; solid squares) to bioreactor / UV disinfected effluent (FEC; open squares). The December 26th influent sample was >300 ng/L and thus, off scale on the above plot.

Ninety-day exposure experiments with goldfish (*Carassius auratus*) in FEC from this plant during Jan-April, 2006 showed significant 4-fold increase in plasma vitellogenin from day 7 to days 21-90 (Jen Kerr, UofA Department of Biology, 2007 - unpublished results). Vitellogenin expression in male oviparous vertebrates may be interpreted as a warning of reproductive consequences (Cheek et al. 2001), and thus, the final effluent produced from the plant sampled may have the capacity to cause reproductive effects in exposed wildlife. Figure 5-3 further illustrates that from mid-September to mid-October was a period during which the effluent wastewater E2-equivalents was the greatest (i.e. 106-175 ng/L). Percent reduction in the levels of RYA measured E2 activity (X_t) varied from -234% to 75% and was found to be mildly serially correlated over time (r = 0.11 for X_t vs X_{t-1}) during the period of data collection. The presence of serial correlation in a variable suggests that there is a time relationship in the data and attempts to apply linear regression with an independent variable may lead to the violation of the independence assumption (Durbin and Watson 1950). The RYA measured E2 activity removal rates have been reported to be greater than 70% for the activated sludge and even trickling filtration plants in the UK surveyed by Kirk et al. 2002. However, research from Canada has shown that in-plant production of RYA measured E2 activity is common in secondary treatment plants (Fernandez et al. 2007c; Servos et al. 2005) similar to what has been found in this work.

5.3.3 RYA vs. ambient/operational/regulatory parameters

All the climate, operational and wastewater quality data shown in Appendix XV including effluent temperature, ambient temperature, rain fall, snow, wastewater flow (inversely proportional to HRT), BOD, TSS, ammonia nitrogen, and solids retention time were used in pair-wise correlations with the estrogenic activity reduction data in an attempt to identify any factor(s) which may be related to the removal of estrogenic contaminants (Figure 5-4A). For any significant correlations found, least-squares linear regression was performed with the percent (%) reduction in E2-equivalents as the dependent variable. Since all the variables in this study were collected over a time-series the Durbin-Watson statistic (Durbin and Watson 1950) was employed to test whether or not the residual errors of adjacent cases are correlated. If they are not, then the results of the regression test of significance would still be valid even [illegible] the observations themselves are serially correlated.

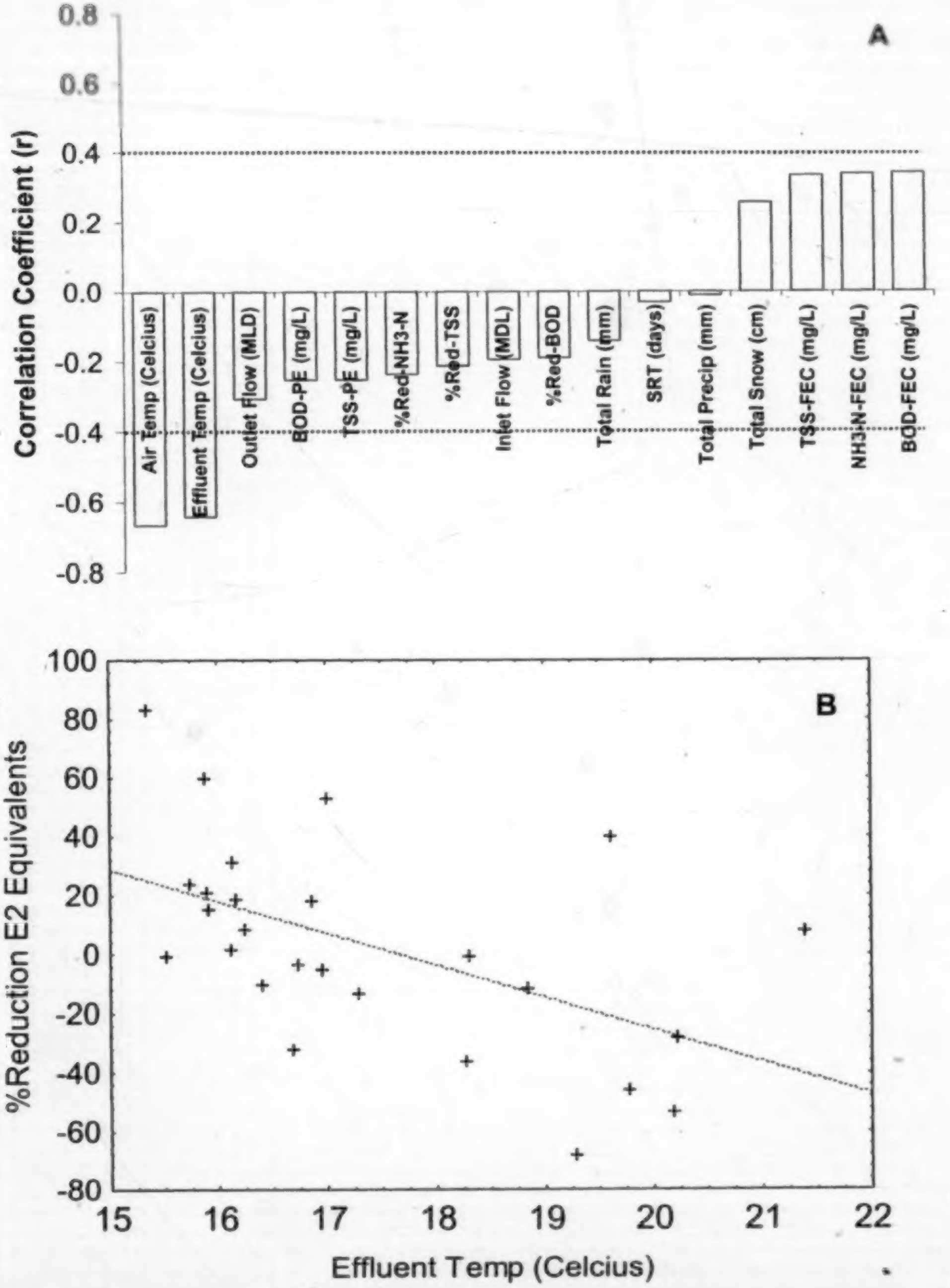

Figure 5-4. Correlation of percentage reduction of RYA measured activity in semi-weekly bioreactor influent and effluent collected from September to December 2006 (A); along with the plot of the significantly correlated variable, effluent temperature (B). Note: hatched line in (A) is the cutoff for 95% significance in correlation; in (B) represents regression line %Red E2-Eq = -10.8·(Eff Temp) + 191, $p = 0.005$, Durbin-Watson $d = 1.97 > d_U = 1.46$, therefore residual error are not correlated (Durbin and Watson 1951).

No correlations were seen in RYA activity reduction with percent reduction in BOD, Flow (i.e. inversely related to HRT), SRT or even rainfall as might be expected. The reduction trends for RYA measured activity were best explained by ambient and effluent temperatures in an inverse fashion. This result was unexpected based on conventional thermally catalyzed decomposition reactions (i.e. microbial growth and enzyme reaction rates increase with temperature). However, upon further examination of the mechanisms of E1 and E2 removal (Fernandez et al. 2007c or Chapter 4), we see that this in fact is a two stage process in most cases, involving first the deconjugation of the bound steroid, followed by the degradation of the free steroid. For E1, which has been identified as the major contributor to RYA measured activity from a suite of 40 eEDCs in the wastewater samples collected from this plant (Fernandez et al. 2007c or Chapter 4), the deconjugation rate (k = 1.6±0.1) was found to proceed faster than the degradation rate (k = 0.77±0.03) in the secondary treatment stage during warmer months. Thus, E1 builds up through the treatment process which corresponds to a build up in RYA measured E2-Eq for the plant sampled. It would appear that during warmer months, more free estrogens which are somewhat recalcitrant are in fact liberated from conjugated form, whereas during colder months little deconjugation occurs. Similar results of temperature related increase in estrogenicity and free estrogen content in the effluent were observed in preliminary work done by this author (see Appendix XVI) for an aerated lagoon treatment system serving a rural population of 20,000 (Site E, Chapter 2 Table 2-1 for WWTP information). Thus, a strong seasonal effect is present in the final effluent concentrations of important eEDCs at municipal wastewater treatment plants.

Whole organism exposure experiments with male goldfish (Jen Kerr, UofA Department of Biology, 2007 - unpublished results) showed the capacity of final effluents to induce a biomarker for detrimental reproductive effects as mentioned previously. The effluent temperatures during this exposure (January to April 2006) ranged from 13-15°C and averaged 14.2°C. This was during the time period found to likely show best reduction potential for eEDCs (Figure 5-4B), compared to warmer months (no exposure data from summer months are available), but even greater reproductive effects are expected to occur in exposed fish during the warmer time periods based on the findings in this work.

When examining the free versus conjugate steroidal estrogen levels (Figure 5-5) in composite (n=4) samples from the warm period (average effluent temperature of 20°C) and one (n=4) from a cold period (average effluent temperature of 16°C), we see that in fact there are more free estrogens produced during bioreactor treatment during the warm period. During the cold period no free estrogens were detected and conjugate estrogens still exist in the final effluent.

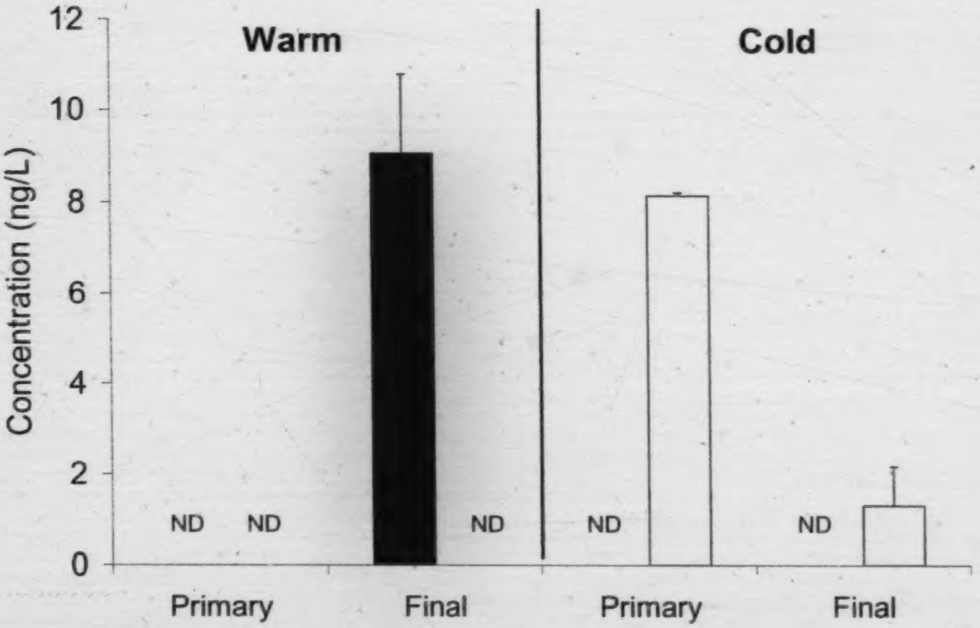

Figure 5-5. Free (E1 only detected; solid bars) versus conjugated (E1-3S, E1-3G, E2-3S detected; open bars) for n=4 composite simples taken during warm period (daily average temperatures: 20°C effluent / 9°C air) and cold period (daily average temperatures: 16°C effluent / -9°C air). Error bars: analytical error (n=3), in standard deviation.

Taken together, the results of this work suggest that despite conditions in the bioreactor, the removal process is dictated by a seasonal dependent cleavage of conjugated estrogens. Summer conditions (wastewater temperatures 18-22°C) seem to result in in-plant production of estrogenicity and E1, whereas winter conditions (wastewater temperatures of 15-18°C) seemed to result in no net accumulation of E1, however, conjugated estrogens would be present in the final effluent.

Labadie and Budzinski 2005 reported that the concentrations of the estrogens most commonly detected in French wastewater effluent and receiving environments exhibited seasonal variations. In the summer, the apparent decay rates of estrogens concentrations exceeded that of dilution, indicating high removal rates from the water column, with 50% of the initial amount of estrone being degraded within 1.7 km downstream of the effluent discharge. In winter however, estrone [1.4] concentrations (1.8 to 1.9 ng/L) did not decrease significantly over a 10 km reach downstream of the effluent discharge. Taken together with the seasonal trends in wastewater estrogenicity reduction/formation presented in this work, it may seem that during the summer months, natural attenuation in the receiving environment may play a greater role than biological wastewater treatment in the removal of problematic eEDC, estrone [1.4] from municipal wastewater. However, in the winter months, the effluent discharged from the plant surveyed may contain little or no estrone [1.4] in free form, nevertheless, significant amounts of estrogen conjugates are being released into the receiving environment. Effluent during the winter temperature regime was still capable of significantly inducing vitellogenin in experimentally exposed goldfish, however, the tank water in these experiments may have been similar to those achieved during summer conditions. Ultimately, seasonal/climate variables along with the sensitivity / exposure of wildlife to eEDCs may be significant factors in predicting the risk to aquatic organisms from municipal wastewater derived eEDCs.

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Chapter6- Summary and General Discussion

6.1 Chapter Summaries

6.1.1 Chapter 1

Several estrogenic endocrine disrupting chemicals (eEDCs) including industrial chemicals, natural and synthetic steroidal estrogens, and various naturally occurring phytoestrogens have been identified as priority organic pollutants with major source inputs being municipal wastewater treatment plant (WWTP) effluents. WWTP point source inputs have been associated with adverse physiological effects including intersex and full sex reversal in fish living down stream. Thus, estrogenic compounds along with several other related compounds have been the focus of much of the chemical and bio-analytical work performed to assess the potential exposure of aquatic organisms to environmental estrogens from municipal and industrial wastewaters. Many estrogenic compounds have been reported to illicit adverse biological effects at concentrations as low as 1 ng/L. Generally, GC-MS and LC-MS methods have been employed to determine the levels of target eEDCs in various environmental samples including wastewater with method detection limits ranging from 0.1 ng/L to 220 ng/L. Recombinant yeast screens (RYAs) which integrate the human estrogen receptor (hER) among others have been developed to detected environmental estrogens in complex matrices. More elaborate bioassays using animal cell lines, as well as whole organism (i.e. small fish, rodent, etc.) bioassays have been developed which may provide more biologically relevant information. However, this gain in biological accuracy is normally accompanied by a loss in endpoint precision due to the inherent variability in dealing with individual organisms compared to tissue / cell cultures. Ultimately, if a large discrepancy is found between chemical and bio-analytical methods for the same sample then a toxicity identification and evaluation (TIE) procedure should be applied to the sample to elucidate the source of the estrogenicity unaccounted for via the chemical method. This will ensure that only relevant chemicals are targeted in the chemical method.

6.1.2 Chapter 2

A suite of 30 primarily estrogenic organic wastewater contaminants was measured in several influent/effluent wastewater samples from four municipal wastewater treatment plants and effluents from one bleached kraft pulp mill (BKME) using an ultra-trace analytical method based on gas chromatography - high resolution mass spectroscopy (GC-HRMS). *In vitro* recombinant yeast assay detection of the E2-equivalents on whole and solid phase extracted (SPE) and fractionated wastewater was also performed. 19-Norethindrone [2.1] was the most frequently detected and abundant (26-224 ng/L) of all the synthetic estrogens/progesterones in the influent samples. 17α-Ethinylestradiol [1.6] was the more frequently detected synthetic estrogen/progesterone in the effluents occurring at or below 5 ng/L with some sporadic occurrences of up to 178 ng/L. The greatest levels of steroidal estrogens in municipal effluents were E1 [1.4] > E2 [1.1] > E3 [1.5] which were all <20 ng/L. Nonylphenol [1.2] and di(2-ethylhexyl) phthalate [2.18] were found to be the highest non-steroidal synthetic compounds surveyed in both municipal influent and effluent samples, both occurring at 6-7 μg/L in municipal effluents. BKME contained relatively large amounts of the plant sterol stigmasterol [2.9] (4 μg/L) but low amounts of fecal sterols, and steroidal estrogens (E2 [1.1] only at 6 ng/L) when compared to the municipal effluents.

In vitro E2-Eq in the wastewater surveyed ranged from 9-106 ng E2/L and ranked from municipal influent>municipal effluent≈BKME, with most of the estrogenicity fractionating in the 100% methanol (mid-polar) SPE fraction followed by a secondary amount in the diethyl ether (low-polar for municipal) or methyl tertiary butyl ether (low-polar for BKME) SPE fractions. Most correlations between chemical and *in vitro* E2-equivalents were weak ($p>0.05$ in most cases). Unexpected inverse correlations between *in vitro* estrogenic activity and concentrations of the estrogenic contaminant bisphenol A [1.3] were found which likely contributed to the weakness of these latter correlations.

A modified toxicity identification and evaluation (TIE) procedure was continued with the SPE extracts from the more potent 100% methanol SPE fractions of municipal effluent. High performance liquid chromatography fractionation with *in vitro* estrogen detection indicated that steroidal estrogens such as E2 were responsible for most of the estrogenicity of the samples. Subsequent collection and GC-MS analysis of active bands did not confirm the presence of steroidal estrogens, but expanded the possibility of phthalate esters (i.e. dibutyl phthalate [2.25]) and natural sterols (i.e. β-sitosterol [1.9]) contributing to the overall estrogenic load.

6.1.3 Chapter 3

A trickling filter/solid contact (TF/SC) biological secondary treatment plant with chlorine disinfection serving a suburban population of 740,000 included in the original survey was further assessed for eEDCs and potential impacts to a target species, chinook salmon (*Oncorhynchus tshawytscha*). Weekly grab samples were taken at established sampling points and analyzed for various pertinent environmental estrogens including industrial chemicals, and natural and synthetic steroidal estrogens. Additionally, human estrogen receptor (hER) activity and capacity to elicit intersex/sex reversal for the wastewater was monitored using a recombinant yeast assay and whole fish exposures, respectively. In this work, the chemical estrogenic equivalent factors or relative potencies for the eight pertinent eEDCs measured via a similar *in vitro* hER recombinant yeast screen were used to generate a cumulative estrogen equivalent (EEq). This total chemical EEq for each sample represented, on average, 9 and 37% of the hER activity in the sample for the primary and final effluents, respectively for the entire sampling period. Linear relationships between chemical EEq and hER ($r = 0.73\text{-}0.75$; significant at 90-95% confidence) were observed for the samples. These observations support the hypothesis that the targeted chemicals in this work were responsible for a significant amount of the estrogenicity in the whole samples assessed.

hER activity levels varied from76 to 106 ng/L E2-equivalents in the primary effluent, and were reduced by 25% by biological treatment. For the primary and final effluent no evidence of sex reversal or intersex was apparent in any of the treatment groups (1%, 3%, 10%, or 100%) based on genetic sex determinations and histological examination of the gonads in alevin from 28 d exposed chinook salmon eggs. However, the possibility that the sexual differentiation of some sensitive individuals in a population could be impacted by exposure to low levels of primary effluent cannot be excluded at this stage given the very low incidence of intersex noted in previous studies with wastewater from the same TF/SC plant.

6.1.4 Chapter 4

A seven-day multi-point sampling campaign based on flow-proportional 24-hour composites allowed for a detailed and accurate examination of the occurrence, in-plant production, and removal rates of eEDCs at each treatment stage (primary, secondary and tertiary) of a municipal WWTP. State-of-the-art eEDC monitoring tools, including, gas chromatography - high resolution mass spectrometry (GC-HRMS), liquid chromatography coupled to triple quadrupole mass spectrometer (LC-MS/MS), and a recombinant yeast assay (RYA) combined with rapid same-day sample preparation techniques were used. Up to 40 eEDC compounds and *in vitro* estrogenicity (in E2-equivalents) of three different polarity (i.e. low, mid and high polarity) fractions was determined for each sample.

Both primary and secondary treatment stages play significant roles in the reduction of several eEDCs. In some cases nearly 100% removal was seen for the natural steroids progesterone [4.9] (99.6%), testosterone [2.7] (99.5%) and estriol [1.5] (98.1%); plant sterols (i.e. Stigmastanol [2.10]; 95.5%); and to a lesser extent bisphenol A [1.3] (90.1%). Less than satisfactory reduction was seen for estradiol [1.1] (62.7%), estrone [1.4] (-130% - "created") and their parent glucuronide and sulfate conjugates [1.18, 1.19, 4.2, 4.3] (70.4%), and for nonylphenol [1.2] (62.2%). In plant production of RYA measured E2 activity was seen at every treatment stage including UV. In addition, estrone [1.4] showed in-plant production during both primary and secondary treatment stages, whereas a 45±36% (95% confidence interval) reduction in residual estrone [1.4] in the effluent was observed for the UV disinfection stage. The relative removal-via-sludge rates versus degradation-in-solution rates were shown for significant eEDCs during both primary and secondary treatment for the seven-day sampling period. Sludge may be an important removal mechanism for some of the eEDCs during primary treatment (as many substances may have been associated with the solids from their initial entry into the sewers), however, sorption to sludge is minimal during secondary treatment, and only slightly significant for the most hydrophobic eEDCs measured such as the plant and animal sterols.

The data obtained from a municipal WWTP were fit to an existing mechanistic model presented in Joss et al. 2004 to obtain kinetic (k_{bio} for deconjugation and degradation) and equilibrium (K_D, solids-liquid partition coefficient) constants for the natural steroidal estrogens know to pose the greatest risk to the reproductive health of aquatic organisms (i.e. E1 and E2).

Pseudo-first order reaction rate constants (k) and solid-liquid partitioning (K_D) coefficients.

Compound	*k_{bio1} (deconjugation)* *L / gSS*d*	*k_{bio2} (degradation)* *L / gSS*d*	*K_D (primary / secondary)* *L / gSS*
E1	0.46±0.03	0.22±0.01	0.35±0.08 / 0.13±0.03
E2	0.70±0.02	1.00±0.08	0.17±0.01 / 0.07±0.01

Note: mean sampling temperature of 15°C; error is ±1 standard deviation from Monte Carlo simulation

The calculation of kinetic constants in this work allowed a quantitative assessment of the pathway specific removal intensity for the most important eEDCs at a full scale BNR plant. The relative rates of deconjugation versus degradation, were greater for E1 than for E2 providing a direct mechanistic explanation as to why E1 was found to accumulate within the treatment works (i.e. since rates of deconjugation proceeded faster than that of degradation making the latter a bottleneck in the elimination of hormonal activity). The trend in the solids liquid partitioning coefficients were E1>E2, and primary > secondary for both E1 and E2, which agrees well with the literature which reports on these values for eEDCs (Ternes and Joss 2006). This means that E1 has the greatest potential for adsorption to primary sludge, however the K_D is still at or below 0.3 which was suggested by Ternes and Joss 2006 as the cut-off for significant adsorption to sludge even at very high sludge production rates. Thus, sorption to sludge (particularly secondary sludge) is not a significant removal mechanism for the most potent eEDCs in municipal wastewater treatment. However, E1 destruction in further processing of primary sludge should be further examined in order to determine any potential risks to human health and the environment via the sludge route for this contaminant.

6.1.5 Chapter 5

The goal of this work was to apply a well established and widely used RYA to assess the reduction efficiency of a full-scale BNR plant for this biologically relevant parameter and eEDC concentrations related to this parameter. Complementary instrumental analyses for targeted eEDCs were also performed in strategic samples. These temporal data (collected from September to December) were combined with operational, wastewater quality, and climate data to determine which of these variables may be related to the levels and reduction of RYA E2-equivalents, intended as a surrogate measure of important eEDCs. Significant variability was present in operational and wastewater quality parameters throughout the sampling period including a 43°C difference in ambient temperature. Most of the wastewater samples collected during this period show a considerable amount of RYA activity with the greatest activity (E2-equivalents of 106-175 ng/L) seen in the final effluents collected from mid-September to mid-October. Percent reduction in the levels of RYA measured E2 activity varied from -234% to 75%. All the climate, operational and wastewater quality data including effluent temperature, ambient temperature, rain fall, snow, wastewater flow (inversely proportional to HRT), BOD, TSS, ammonia nitrogen, and solids retention time were used in pair-wise correlations with the estrogenic activity reduction data in an attempt to identify any factor(s) which may be related to the removal of estrogenic contaminants. No correlations were seen in RYA activity reduction with percent reduction in BOD, Flow (i.e. inversely related to HRT), SRT or even rainfall, and the reduction trends for RYA measured activity were explained best by ambient and effluent temperatures in an inverse fashion (%reduction in E2-equivalents = -10.8·(effluent temperature in °C) + 191, $p = 0.005$). The concentration of free versus conjugate steroidal estrogen levels in composite samples from a summer period ($n=4$; average effluent temperature of 20°C) were compared with that from a winter period ($n=4$; average effluent temperature of 16°C). It was found that more free estrogens were produced during bioreactor treatment in the summer-like conditions.

6.2 General Discussion

The objectives of this authors Ph.D. work as stated in Chapter 1 section 1.2 were as follows (slight modifications added in this section shown underlined):

1. Perform a survey of estrogenicity / potential biological effects and individual concentrations of known potent eEDCs, and if needed toxicity identification and evaluation (TIE) assessment of effluents collected from various wastewater treatment plants in Canada.

2. Refine and optimize analytical methods for the detection of priority eEDCs identified in objective 1 both in dissolved and adsorbed forms, as well as, develop a suitable method to detect conjugated eEDCs dissolved in wastewater.

3. Perform an in-depth mass balance and seasonal/temporal monitoring of priority eEDCs along with net estrogenicity in select treatment plant(s) to assess unit process/operation removal efficiency for key eEDCs and to determine which if any ambient and operationally controlled factors may affect this removal efficiency.

6.2.1 Biological effects (Objective 1)

In the introductory chapter, the biological / toxicological significance of eEDCs in the environment is presented as the major driving force for further eEDCs research. In the present thesis research, Chapter 3 presents a whole organism toxicological evaluation for the impact of eEDCs from one of the WWTPs surveyed on the sexual development of Chinook salmon from eggs to alevin. Environmentally relevant concentrations of municipal effluent were tested however, for all sub-lethal doses (i.e. ≤10%) of secondary effluent performed, no intersex or sex reversal effects were detected in any of the fish (n=100 performed in duplicate exposures for each exposure concentration). However, in previous experimental trials with the same effluent and species of salmon Afonso et al. 2002 found one intersex fish and two sex reversals in the chinook exposed to 30% secondary effluent (24 genetic males assessed), and one intersex fish in the group exposed to 100% secondary effluent (6 genetic males assessed), and no intersex or sex reversal in the fish exposed to 10% secondary effluent (20 genetic males assessed) which corroborates our work for this latter exposure concentration. Thus, >10% effluent concentrations would be required to produce intersex / sex reversal effects in a predominant target fish species for site B (Trickling filtration plant). The percentage (%) reduction in RYA measured estrogenicity for this plant was 25% (to 67 ng/L E2-equivalents in the final effluent), however, both E1 [1.4] and E2 [1.1] where found to increase in the effluent during treatment by 370% and 11% to mean concentrations of 41 ng/L (ND-147 ng/L) and 6 ng/L (ND-13 ng/L), respectively, as assessed over the eight week sampling period (see Appendix IV, site B Infl-8 and Effl-8). In comparison, for the plant focused on in Chapter 4 work (site D - a full BNR facility with UV disinfection) RYA measured estrogenicity was found to increase by almost 300% (to a seven-day mean of 207 ng/L), with E1 [1.4] increased by 130% and E2 [1.1] decreased by 62% and mean final effluent concentrations of 65 ng/L (37-93 ng/L) and 20 ng/L (10-129 ng/L), respectively. Thus, the sexual disrupting potential of this effluent (i.e. site D) may be greater than that of site B which was assessed for intersex and sex reversal effects in fish in this work.

Site D was assessed for a biomarker of sexual disruption, vitellogenin in male goldfish (*Carassius auratus*) exposed to 100% FEC from this plant during Jan-April, 2006 (Jen Kerr, 2007 – unpublished results). Significant 4-fold increase in Vtg (n=10 male fish per exposure) compared to controls (n=10) exposed to only tap water were found from day 7 to days 21-90. Palace et al. 2002 performed a whole lake (Lake 260) exposure experiment with concentrations of synthetic estrogen EE2 [1.6] ranging between 4.0 and 8.1 ng/L. Male fathead minnows (*Pimephales promelas*) captured from Lake 260 after EE2 [1.6] additions began contained 9000-fold higher concentrations of Vtg, than were detected in fish captured from the same lake prior to the EE2 [1.6] additions. Additionally, EE2-exposed male fatheads in Lake 260 showed widespread fibrosis and inhibition of testicular development, enlargement of liver cells, edema in the interstitium between kidney tubules, and eosinophilic deposits in the kidney tubule lumen. Ultimately, Vtg induction in males compromises kidney function and leads to scarring and tissue death in the testes. Thus, there is sufficient evidence to suggest that effluents from site D have the capacity to cause adverse effects in sensitive species (i.e. fathead minnows) at ecologically relevant concentrations (~10%), as the E2-equivalents of the Lake 260 exposure would have been at least 4-7 ng/L (88.8% estrogen equivalents factor for EE2 [1.6]), and a 10% dilution of site D effluent would have an E2-equivalents of 20.7 ng/L.

6.2.2 Analytical methodology (Objectives 2)

6.2.2.1 Instrumental Methods

A comprehensive gas chromatographic – high-resolution mass spectrometric (GC-HRMS) based method was developed (with the help of this author) that permitted the simultaneous determination of 30 estrogenic endocrine-disrupting chemicals (EDCs) and related compounds including surfactants, biogenic and synthetic steroids, fecal sterols, phytoestrogens, and plasticizers in wastewater (Ikonomou et al. 2007). Features of the method include low sample volume (~40 ml) optimized Florisil® clean-up to minimize matrix interferences and optimized analyte derivatization to improve sensitivity via GC-HRMS. Method detection limits (MDLs) ranged from 1 ng/L to 0.5 μg/L, and were 7.1 ng/L for E2 [1.1], 7.6 ng/L for E1 [1.4], 1.5 ng/L for E3 [1.5], 7.1 ng/L for EE2 [1.6], 2.1 ng/L for BPA [1.3] and 0.2 μg/L for NP [1.2]. However, considering biological effects may be apparent with eEDC exposures as low as 1 ng/L (EE2), lower MDLs for these important eEDCs were sought. Also, methods for the analysis of conjugated estrogens, not suitable for GC based analysis due to their lack of volatility, were needed if a complete mass balance was to be performed for these important steroidal estrogens (i.e. E1, E2, E3 and EE2). Additionally, an accurate and robust sample preparation technique was needed for the assessment of eEDC sorbed to major solid constituents in the wastewater treatment process. The GC-HRMS method was suitable for the initial survey as it allowed for the simultaneous determination of a large number of analytes. However, for the mass balance work, greater sensitivity and precision was obtained by turning to a liquid chromatography – electrospray ionization (ESI) technique. LC-ESI-MS generally allows for a simplified sample preparation for aqueous samples and high sensitivity for low volatile, mid-polar substances (i.e. many of the analytes in this work) as compared to GC-MS (Petrovic et al. 2002). For this work a LC-MS/MS (API 5000 system) was available and provided MDLs of less than 1 ng/L (based on a 100-300 mL sample size) for the most significant eEDCs in this work. However, the more lipophilic eEDC compounds, including the plant and animal sterols β-sitosterol [1.9] and coprosanol [1.15], showed very poor sensitivity via LC-MS/MS and these substances were still measured using the GC-HRMS technique.

Table 6-1. Comparison of triplicate spike precision (relative standard deviation - %RSD) between GC-HRMS and LC-MS/MS methods for key eEDCs in municipal wastewater effluent.

Compound	GC-HRMS (%RSD)[a]	LC-MS/MS (%RSD)[b]
E1 [1.4]	10	11
E2 [1.1]	6.3	12
E3 [1.5]	60	17
EE2 [1.6]	7.7	10
NP [1.2]	33	28
BPA [1.3]	34	22

[a] Ikonomou et al. 2007; [b] Normally distributed SD from Appendix XIV.

In the mass balance work, eEDC adsorbed to solids (>16 μm which was the smallest pore size providing reasonable gravity filtration rates for same-day sample preparation) and dissolved eEDCs were analyzed separately. As for most wastewater processes the minimum settleable solids cut-off is generally around 10-25 μm (Tchobanoglous et al. 2003) such that the material classified as "solids" in our work was a functional definition adhering to the requirements of the mass balance assessment (i.e. removal in the sludge versus degradation in the water column). The sample preparation techniques developed for the mass balance / seasonal variability work (Chapters 4 and 5), included SPE extraction of the liquid fraction and accelerated solvent extraction (ASE) of the solids to replace the liquid-liquid extraction of the whole effluent previously used with the GC-HRMS technique. The new protocol is presented in greater detail in Chapter 4, where it is shown that the sensitivity and accuracy of these methods meet the needs of the study. Table 6-1 shows that the precision via LC-MS/MS tends to be more consistent, and higher in most cases when compared to GC-HRMS analysis for the same analytes.

6.2.2.2 Bio-Analytical Methods

RYA proved to be a fast (several hundred samples could be analyzed in two working days, with a weekend incubation), reproducible (<10% RSD) and sensitive (MDL of 4-8 ng/L E2) tool to assess complex mixtures from wastewater processes for the presence of eEDCs (See Appendix III for more RYA methodology details). The major downsides of using RYA to assess reproductive disruption potential in wastewater were due to the fact that this assay shows a basal expression of hER activity even in the absence of ER agonists, as well as, the fact that this assay represents a relatively simplistic biological system with limited metabolic capabilities (Soto et al. 1995). However, in this work, we have established both in Chapters 3 and 4 a statistically significant positive correlation between major eEDC levels (i.e. E1, E2, NP) and RYA measured estrogenicity. In Chapter 3 we see that 9-37% of the hER activity measured by RYA could be explained by the targeted chemicals in this work (namely E1 and E2). In the introductory chapter, we estimated that 57-93% of the typical RYA and E-screen measured estrogenicity from treated municipal effluent was unexplained which agrees with our results. In Chapter 2, we saw that there were complexities in relating the bioassay results to the chemically determined EEq since BPA may have been acting as a hER antagonist in the presence of potent estrogens. Additionally, Chapter 3 section 3.3.1 lists potential explanations for the large discrepancy between chemical estimation of estrogenicity (assuming additivity of estrogenic equivalency) and observed net estrogenic activity *in vitro*. In order to complement the RYA results in this work with a mammalian cell system, the E-screen (Soto et al. 1995) was attempted but no assay culture could be grown since the ATCC MCF-7 cells obtained showed very poor growth characteristics (Villalobos et al. 1995). Nelson et al. 2007 showed that E-screen corresponded well with two different RYAs including the RYA system used in this work (Routledge and Sumpter 1996) and one developed by Gaido et al. 1997 for whole effluents sampled from a range of municipal WWTPs in and around Vancouver, BC.

6.2.3 Toxicity identification and evaluation (TIE) (Objective 1)

A modified toxicity identification and evaluation (TIE) protocol was followed with effluents surveyed in Chapter 2 due to the large discrepancy seen between the RYA measured E2-equivalents and the chemical EEq as discussed in section 6.2.2.2. In accordance with USEPA's national pollutant discharge elimination system (NPDES), TIE is a protocol performed in three phases: toxicity characterization (Phase I), toxicant identification (Phase II) and toxicant confirmation (Phase III) (Anon. 1999). Phase I characterizes the types of effluent toxins by testing the toxicity of aliquots of effluent samples that have undergone bench-top manipulations (i.e. filtration, SPE fractionation in our case). Phases II and III involve further treatments in conjunction with chemical analysis (e.g. GC-MS) to identify the compounds causing effluent toxicity. In our case, Phases I and II were performed with RYA as the ultimate toxicity test for reproductive disruptors / eEDCs. Although effluents and in some cases influents from all five plants surveyed in Chapter 2 (sites A, B, C, D and E) were run through phase I and II TIEs, only site B results are presented since effluents from this plant showed the greatest levels of eEDCs and associated estrogenicity, and many negative results were obtained in the TIE screening due to the difficulty of obtaining sufficient amounts of material after extensive chemical fractionations (i.e. SPE, HPLC). As described in great detail in Chapter 2 section 2.3.4, several abundant organic chemicals, some of which were currently targeted via the GC-HRMS method applied, and some which were not, were identified in wastewater effluents via detailed HPLC fractionation and subsequent GC-MS analysis of the RYA active fractions. Ultimately, the results suggested that the bulk of the estrogenicity in the municipal wastewater which is thought to be due to steroidal estrogens may also be in part due to high levels of phthalate esters and natural sterols in the wastewater. In the mass balance / seasonal variation work, the analytical methodology targeted phthalate esters (i.e. DEHP [2.18]), sterols (i.e. plant and animal sterols), along with several important natural and synthetic estrogens and their conjugates (see Table 4-1 in Chapter 4). The compounds on the target substance list (Table 4-1) were also selected based on their frequency of occurrence in the survey study (Chapter 2) along with relationships to RYA activity and known adverse effects on wildlife (Chapter 3).

Ultimately, the goal of any TIE program and related chemical analysis is to identify those substances which should be prioritized in future studies. In addition, when TIE is applied to municipal effluents, toxicity reduction is also one of the main desirable outcomes in the identification of the toxicant. Thus, in this research, we have followed this general USEPA structure, and continued towards a "toxicity reduction program" where we attempt to acquire more in-plant data on the priority chemicals which may pertain to their elimination during treatment.

6.2.4 eEDCs in WWTPs (Objective 3)

6.2.4.1 Reduction efficiency and related factors

The reduction efficiency of several pertinent eEDCs in wastewater treatment have been published by various researchers and summarized in Chapter 1, Table 1-4. E1 [1.4], NP [1.2] and plant sterols (e.g. β-Sitosterol [1.9]) all show inadequate removal rates (<90%) compared with other eEDCs. In this work, some of the preliminary survey work presented in Chapters 2 and 3, show that these substances including the synthetic estrogen EE2 [1.6] were not effectively removed by a range of treatment types, including lagoon, trickling filtration and activated sludge plants based on their lowest observable effects concentrations (LOELs) (see Table 3-1 in Chapter 3). The order for general reduction efficiencies for eEDCs, via different types of treatment processes published in the literature was: membrane biological reactors (MBRs) > AST with nutrient removal > AST without nutrient removal > trickling filtration (Drewes et al. 2005; Shi et al. 2004; Kirk et al. 2002; Joss et al. 2004; Andersen et al. 2003). However, in this work, we do not generally see this trend for the plants sampled. No MBRs were sampled; however, AST with nutrient removal (sites C and D) and a trickling filtration plant (site B) were extensively sampled in this work. We have compared the reduction efficiency from these two plants in section 6.2.1 with respect to RYA, E1 and E2 levels, where no obvious performance improvements were seen for these parameters in site D versus site B. However, time of sampling may be an issue as was demonstrated in Chapter 5, with the summer months (time when AST site D was sampled) showing greater effluent RYA and free estrogens levels compared to winter months (when TF site B was sampled). Ultimately, as the activated sludge process with nutrient removal, including new membrane bioreactors, is increasingly becoming the most popular method of choice for treatment of municipal wastewaters in larger municipalities (>50,000 population equivalents) we chose to focus the detailed mass balance work on a BNR plant (i.e. site D).

The results in Chapter 4 also contradict the general consensus in the literature that a common municipal WWTP with an activated sludge system for nitrification and denitrification including sludge recirculation can appreciably eliminate natural and synthetic estrogens (Andersen et al. 2003). There are certain reports of activated sludge secondary treatment leading to removal rates of >85% for natural estrogens E1 [1.4] and E2 [1.1] from Europe and the US (Andersen et al. 2003; Drewes et al. 2005). Also, it is suggested that nitrification/denitrification processes aids in the removal of steroidal estrogens and associated estrogenicity, however, the relationship may have more to do with better growth conditions (Servos et al. 2005) and possibly an increase in solids retention time (SRT) associated with these advanced processes, and not necessarily the bacteria generally associated with nitrification (e.g. *Nitrosomonas europaea*) (Shi et al. 2004).

In this work (i.e. Chapter 5), as with most published studies on the topic, no statistically significant relationship was seen between SRT or HRT and eEDC removal, however it was generally observed that plants with greater SRTs (i.e. > 10 days at 10°C) show better EDC removal rates (Clara et al. 2005; Hashimoto et al. 2007; Servos et al. 2005). Site D (subject of Chapter 4 and 5) had a SRT of 7 to 8 days in the winter and 4 to 5 days in the summer and thus did not fall into the category of plants with good EDC removal rates. Ultimately, this author's work presented in Chapter 5 (see Figure 5-3B) has demonstrated that a statistically significant inverse relationship exists between wastewater temperature and RYA measured estrogenic removal efficiency. Temperature influences metabolic activities of microorganisms, has a large effect on gas-transfer rates and settling characteristics of biological solids (Tchobanoglous et al. 2003) and thus, it is not surprising that it may play an important role in the removal efficiency of micropollutants such as eEDCs. The reasons for the inverse relationship between RYA E2-equivalnets (and related E1 and E2) removal and wastewater temperature are discussed in detail in section 5.3.3. A similar effect was observed by this author in some preliminary work done on eEDCs and RYA activity in aerated lagoon (site E) effluents as seen in Appendix XVI, thus, further confirming this effect as significant. No effects of this type have been noted in the literature and these findings are considered novel.

6.2.4.2 Mass Balance and Kinetics

In Chapter 1, equation (1-1) is used to illustrate the mass balance of a particular pollutant (*x*) in a bioreactor. This general equation is used to calculate the flux of contaminant that came into the reactor to different compartments of the process, including adsorption and removal in excess sludge, degradation in the bioreactor or exiting the reactor in the effluent. As only three of the four components (i.e. input, output, accumulation, degradation) of the mass balance could be determined in our research on a full-scale facility, the degradation rate had to be calculated as the remaining quantity. As shown in Figure 4-5, the results of the mass balance work on natural steroidal estrogens corroborates the observations made by many other researchers. These observations include: estrogen adsorption to excess sludge is minimal (<5% of the total flux); moderate amounts of E2 [1.1] are degraded; and E1 [1.4] is found to be insufficiently removed and may accumulate in the bioreactor effluent (Hashimoto et al. 2007, Servos et al. 2005; Andersen et al. 2003). The kinetic and equilibrium parameters calculated with error estimates and tabulated in Table 4-2 provide intercomparable measures to relate the fate and removal efficiency of these key eEDCs in this cold region BNR facility to other facilities or pilot scale processes. There is no other published work reporting on these parameters for a full scale plant aside from K_D (solids-liquid partitioning coefficient) shown for one full scale plant (Andersen et al.

2003). As for the other parameters found in Table 4-2 comparable data only exists for batch experiments (Joss et al. 2004 and Ternes et al. 1999) which has already been discussed in section 4.3.3. However, here we are illustrating the novel nature of this work as no data of this kind is available for cold region BNR processes which may in fact be much less efficient in the removal of eEDCs than its warmer counterparts.

6.3 Recommendations

6.3.1 Future Research Needs

Future work based on the findings of this research are summarized here based on the major findings and conclusions of this work.

1. Continuation of toxicity identification and evaluation (TIE) protocol (Chapter 2) for the discovery of new sources of estrogenicity with an emphasis on:
    a. Preparative scale HPLC using methods optimized for the separation of hormones, sterols and xenoestrogens
    b. LC-MS/MS or LC-Q-TOF techniques with complementary RYA detection.

2. Batch experiments with activated sludge from Site D (Chapter 4):
    a. Examine factors influencing $k_{deconjugation} > k_{degredation}$
    b. Examine temperature dependence of $k_{deconjugation}$

3. Investigate UV effect on E1 and estrogenicity and characterize intermediates (Chapter 4)

4. Potential seasonal considerations when examining ecological effects in receiving environment from the results for site D.
    a. Summer: Do E1, E2 and NP pose a risk to biota?
    b. Winter: Do conjugated estrogens pose a risk to biota?

6.3.2 Recommendations for Optimization of the WWTPs

1. Microbial community composition of cold environment BNR may not be favorable for the removal of EDCs based on comparisons to the reported performance of BNRs from warmer regions.

2. Enhanced enzyme activity during preliminary or primary treatment may liberate free estrogens providing an extended opportunity for free estrogens to degrade in the bioreactor.

3. UV treatment optimization for the removal of E1 which seems to pose the largest threat to aquatic organisms in the receiving environment.

4. MBRs may show promise for eEDCs, with longer SRT possible, and UF removal of mid-polar to non-polar micropollutants.

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APPENDIX I – Chemical information and structures

Common Name [Number]	**IUPAC/systematic Name**	**MW (g/mol)**	**K$_{OW}$**	**HLC (atm m^{3} / mol)**
17α/β-Estradiol (E2) [1.1]	(17α/β)-Estra-1,3,5(10)-triene-3,17-diol	272.4	3.9^{a}	6x10^{-7a}
Nonylphenol (NP) [1.2]	1-(4-Hydroxyphenyl)nonane	220.4	4.5^{a}	11^{a}
Bisphenol A (BPA) [1.3]	4,4'-(1-Methylethylidene)bisphenol	228.3	3.4^{a}	1x10^{-10a}
Estrone (E1) [1.4]	3-Hydroxyestra-1,3,5(10)-trien-17-one	270.4	3.4^{a}	6x10^{-7a}
Estriol (E3) [1.5]	Estra-1,3,5(10)-triene-3,16,17-triol	288.4	2.8^{a}	2x10^{-11a}
17α-Ethinylestradiol (EE2) [1.6]	(17α)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol	296.4	4.2^{a}	4x10^{-7a}
Equilinin [1.7]	3-Hydroxyestra-1,3,5,7,9-pentaen-17-one	266.3	na	na
Pinosylvin [1.8]	(E)-5-(2-Phenylethenyl)-1,3-benzenediol	212.3	na	na
β-Sitosterol [1.9]	(3β)-Stigmast-5-en-3-ol	414.7	na	na
Genistein [1.10]	5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	270.2	na	na
Glucuronic acid [1.11]	D-glucuronate	194.1	na	na
Sulfate [1.12]	Sulfate	96.1	na	na
Daidzein [1.13]	7-Hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	254.2	na	na
Equol [1.14]	7-hydroxy-3-(49-hydroxyphenyl)chroman	230.3	na	na
Coprostanol [1.15]	(3β, 5β)-Cholestan-3-ol	388.7	na	na
Cholesterol [1.16]	Cholest-5-en-3β-ol	386.7	na	na
Nonylphenol Polyetholxylates (NPEOs) [1.17]	na	264-1100	4.2^{a}	na
17β-Estradiol-3-glucuronide (E2-3G) [1.18]	1,3,5(10)-Estratriene-3,17*β*-diol 3-glucuronide	448.5	na	na
Estrone-3-glucuronide (E1-3G) [1.19]	1,3,5(10)-Estratrien-17-one 3-glucuronide	446.5	na	na
17α-Ethinylestradiol-3-glucuronide (EE2-3G) [1.20]	1, 3, 5(10)-Estratrien-17*α*-ethynyl-3, 17*β*-diol 3-glucuronide	472.5	na	na
19-Norethindrone [2.1]	(17α)-17-Hydroxy-19-norpregn-4-en-20-yn-3-one	298.4	na	na
(-)-Norgestrel [2.2]	13-Ethyl-17-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one	312.5	na	na
Mestranol [2.3]	(17α)-3-Methoxy-19-norpregna-1,3,5(10)-trien-20-yn-17-ol	310.4	4.7^{b}	na
Equilin [2.4]	3-Hydroxyestra-1,2,5(10), 7-tetraen-17-one	268.4	3.4^{b}	na
α-Zearalanol [2.5]	6-(6,10-Dihydroxyundecyl)-β-resorcylic acid-α-lactone	322.4	na	na
17β-Estradiol-3-benzoate [2.6]	(17β)-Estra-1,3,5(10)-triene-3,17-diol-3-benzoate	376.5	na	na
Testosterone [2.7]	17β-Hydroxyandrost-4-en-3-one	288.4	na	na
Campesterol [2.8]	(24R)-Ergost-5-en-3β-ol	400.7	na	na
Stigmasterol [2.9]	(3β, 22E)-Stigmasta-5,22-dien-3-ol	412.7	na	na
Stgmastanol [2.10]	(3β,5α)-Stigmastan-3-ol	416.7	na	na
Desmosterol [2.11]	3β-Cholesta-5,24-dien-3-ol	384.7	na	na
Ergosterol [2.12]	(3β, 22E)-Ergosta-5,7,22-trien-3-ol	396.7	na	na
6-ketocholesterol [2.13]	(3β)-Hydroxy-cholestan-7-one	402.7	na	na
7-keotcholesterol [2.14]	(3β)-Hydroxy-cholest-5-en-7-one	400.6	na	na
Coprostan-3-one [2.15]	5β-Cholestanone	386.7	na	na
Fucosterol [2.16]	Stigmasta-5,24(28)-dien-3-ol	412.7	na	na
Totarol [2.17]	14-isopropyl-podocarpa-8,11,13-trien-13-ol	286.5	na	na
Bis(2-ehtylhexyl) phthalate (DEHP) [2.18]	Bis(2-ehtylhexyl) phthalate	390.6	7.5^{a}	2x10^{-5a}

APPENDIX I (Continued)

Common Name [Number]	**IUPAC/systematic Name**	**MW (g/mol)**	**K_{OW}**	**HLC (atm m^3 / mol)**
Di-n-octylphthalate (DnOP) [2.19]	Di-n-octylphthalate	390.6	na	na
3β-Stigmasta-5,25-dien-3-ol [2.20]	3β-Stigmasta-5,25-dien-3-ol	412	na	na
Galactouronic acid [2.21]	D-Galactopyranuronic acid	194	na	na
Binaphthylsulphone [2.22]	2,2-Dinaphthylsulfone	318	na	na
Terephthalic acid [2.23]	1,4-benzenedicarboxylic acid	166.1	na	na
Guaifenesin [2.24]	3-(2-Methoxyphenoxy)-1,2-propanediol	198.2	na	na
Dibutyl phthalate (DBP) [2.25]	Dibutyl-1,2-benzenedicarboxylate	278.3	na	na
Azelaic acid [2.26]	1,7-Dicarboxyheptane	188.2	na	na
D-(+)-Sucrose [2.27]	α-D-Glucopyranoside-β-D-fructofuranosyl	342	na	na
Diethylstilbestrol (DES) [4.1]	(E)-4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol	268.4	5.1[b]	na
Estrone-3-sulfate (E1-3S) [4.2]	1,3,5(10)-Estratrien-17-one 3-sulfate	350.5	na	na
17β-Estradiol-3-sulfate (E2-3S) [4.3]	1,3,5(10)-Estratriene-3,17β-diol 3-sulfate	352.5	na	na
Estriol-3-sulfate (E3-3S) [4.4]	1,3,5(10)-Estratriene-3,16α,17β-triol 3-sulfate	368.5	na	na
Estriol-3-glucuronide (E3-3G) [4.5]	1,3,5(10)-Estratriene-3,16α,17β-triol 3-glucuronide	464.5	na	na
Estriol-16-glucuronide (E3-16G) [4.6]	1,3,5(10)-Estratriene-3,16α,17β-triol 16-glucuronide	464.5	na	na
17α-Ethinylestradiol-3-sulfate (EE2-3S) [4.7]	1, 3, 5(10)-Estratrien-17α-ethynyl-3, 17β-diol 3-sulfate	376.5	na	na
16α-Hydroxyestrone [4.8]	1,3,5(10)-Estratriene-3,16α-diol-17-one	286.4	na	na
Progesterone [4.9]	Pregn-4-ene-3,20-dione	314.5	na	na

a. Birkett JW, Lester JN. 2003. Endocrine disrupters in wastewater and sludge treatment processes. Boca Raton: Lewis Pub.

b. Ternes TA, Joss A. 2006. Human pharmaceuticals, hormones and fragrances : the challenge of micropollutants in urban water management. London ; Seattle: IWA Pub. xviii, 453 p. p.

APPENDIX I (continued)

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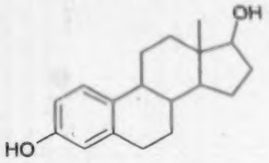

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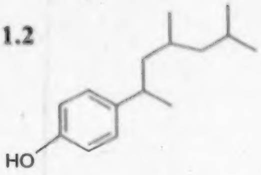

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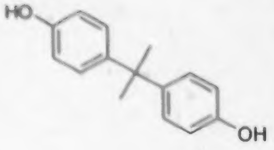

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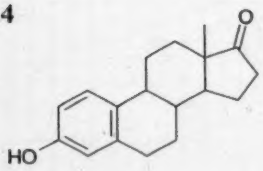

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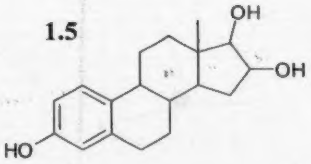

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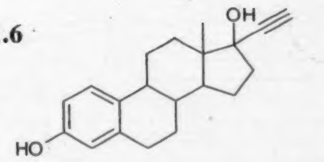

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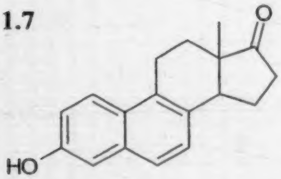

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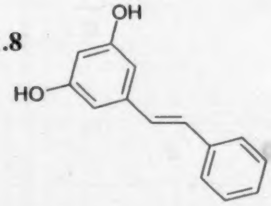

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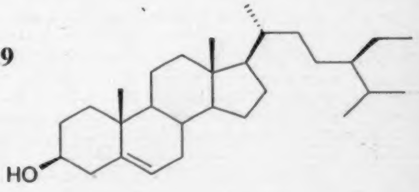

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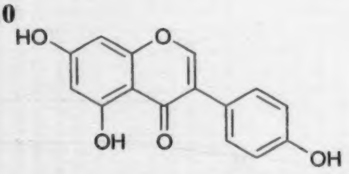

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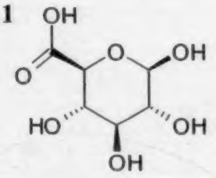

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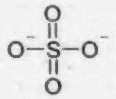

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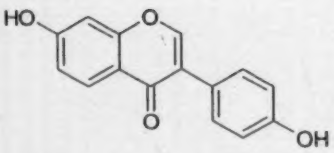

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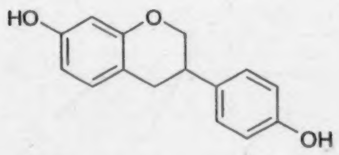

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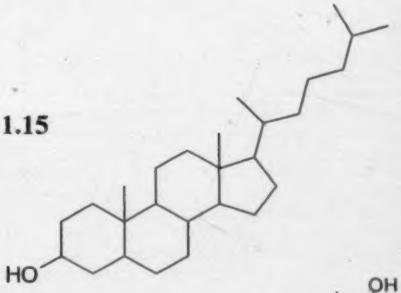

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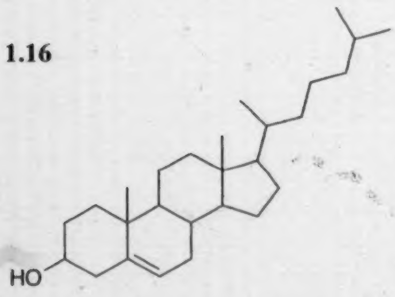

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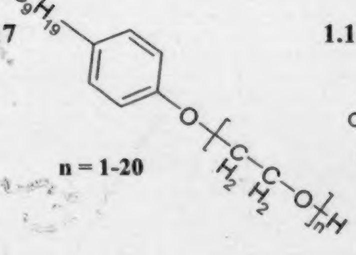

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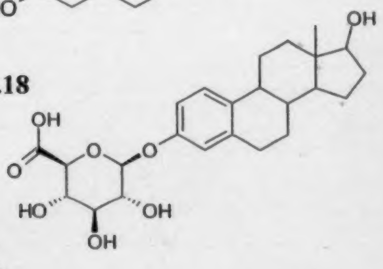

APPENDIX I (continued)

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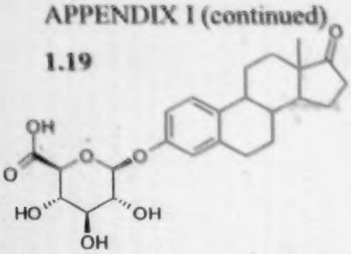

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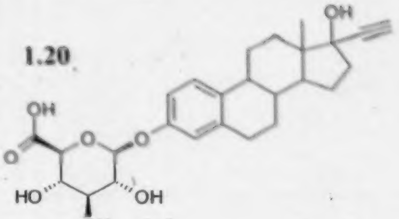

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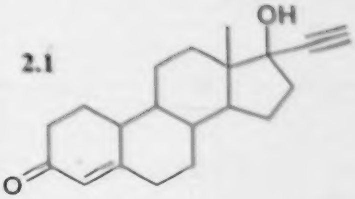

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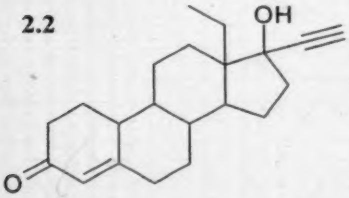

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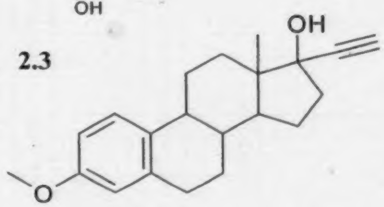

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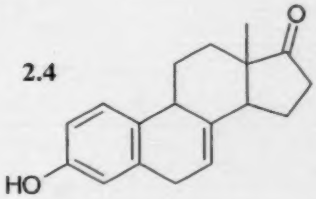

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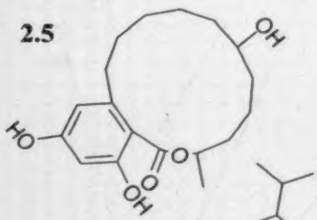

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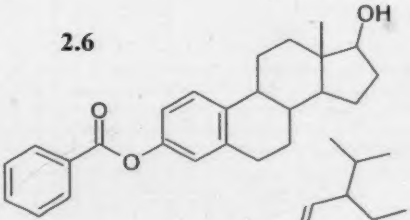

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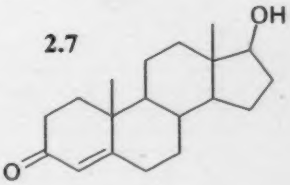

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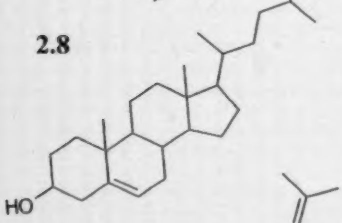

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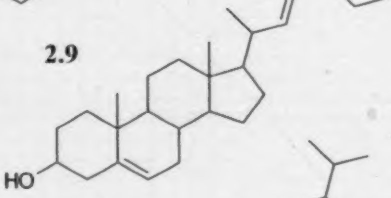

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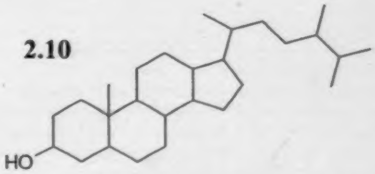

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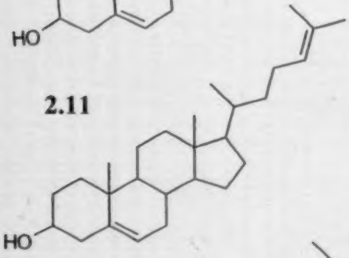

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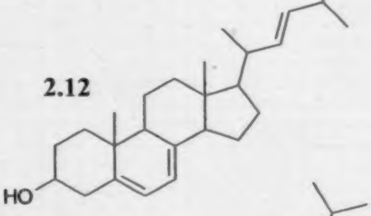

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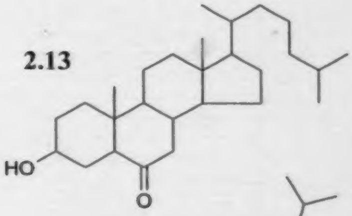

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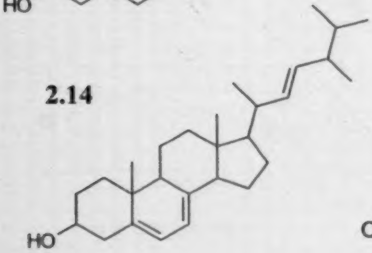

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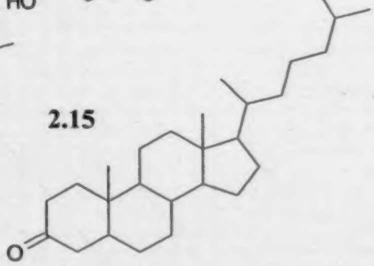

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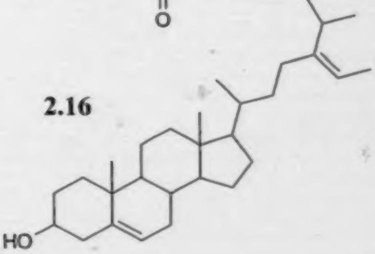

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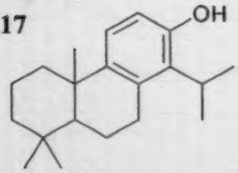

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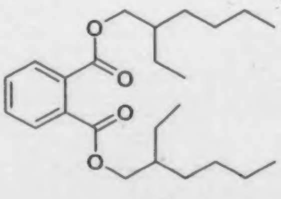

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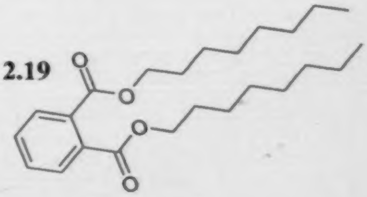

APPENDIX I (continued)

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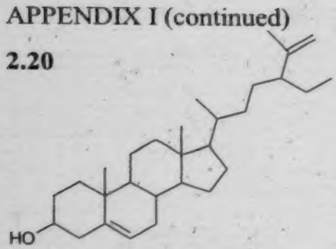

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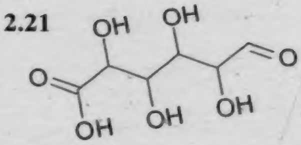

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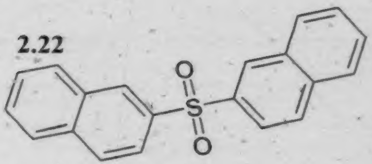

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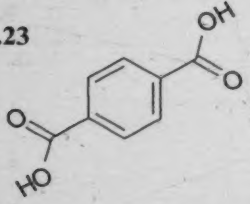

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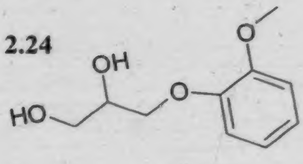

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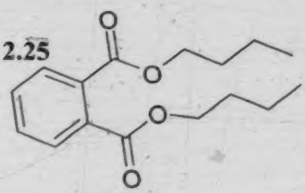

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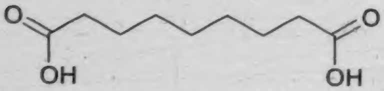

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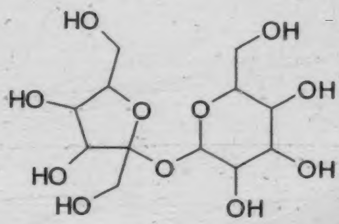

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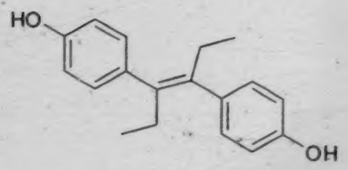

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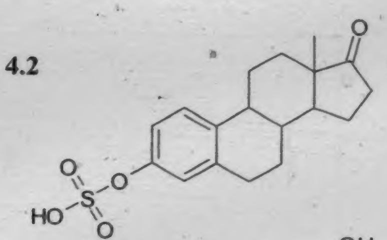

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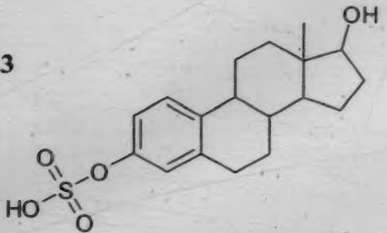

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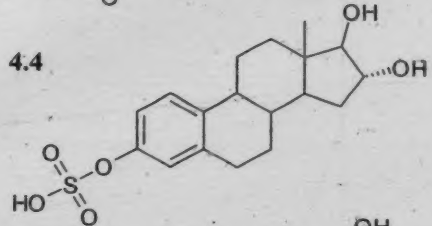

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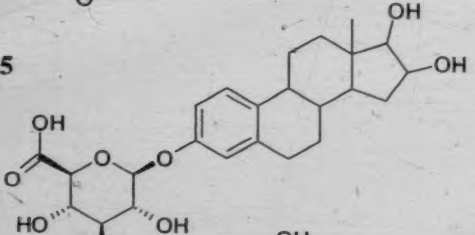

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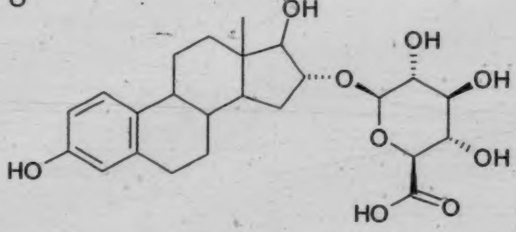

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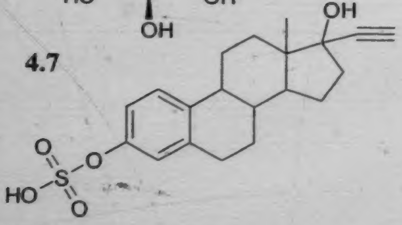

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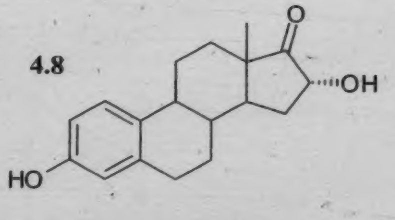

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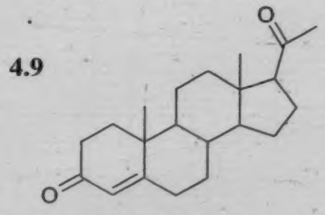

APPENDIX II – GC-HRM methodology details

Preparation of standards

Stock solutions were made up in suitable solvents based on the accurate mass (corrected for reported purity of standards) of analytes. Five 13C-labeled, deuterated or non-naturally occurring internal standards were used for recovery correction for representative analyte concentrations determined. These internal standards were prepared in a similar manner as the analyte standards to make an internal standard mixture in MeOH (see Table A2-1). β-Estradiol-17-acetate has previously been successfully used as an internal standard to quantify steroidal EDCs in municipal sewage. Three deuterated polycyclic aromatic hydrocarbons, phenanthrene-d10, anthracene-d10, and chrysene-d12 (5 μl of ~4 ng/μl each = 20 ng) were added to the extracts prior to GC-HRMS analyses. These were used as performance standards for evaluation of internal standard recovery.

Table A2-1. Internal standard mixture (ISM) and GC-HRMS ions monitored for each compound.

Internal Standard	Compound Class	Concentration in ISM (ng/μL)
Ring-$^{13}C_6$-nonylphenol	A	0.6
Propane-d_6-bisphenol A	B	4.0
Di-n-octylphthalate-d_4	C	10.0
β-Estradiol-17-acetate	D	3.0
2,2,3,4,4,6-d_6-cholesterol	E	10.0

CIL is Cambridge Isotope Laboratories, Andover, MA, USA; Sigma is St Louis, MO, USA.

APPENDIX II – GC-HRM methodology details (Continued)

Instrumental analysis

One microliter of each sample was injected (splitless mode) onto a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled to a VG AutoSpec magnetic sector mass spectrometer (Micromass UK, Manchester, UK). Analytes were resolved on a fused-silica capillary column of stationary phase DB-5 MS (20 m x 0.25 mm inner diameter, 0.25 μm film thickness, J&W, Folsom, CA, USA) with helium as a carrier gas. Samples were injected using a high pressure (150 kPa for 1 min) loading to facilitate the activation of the automatic solvent trip which ensures the filament is off when the solvent reaches the ionization source in the particular instrument used. A purge time of 0.5 min was used and the subsequent pressure program was 150 kPa to 100 kPa in 1 min; 100 kPa to 136 kPa at 4.5 kPa/min; and 136 kPa to 190 kPa at 2.7 kPa/min with a final hold of 2 min. Splitless injection with a temperature of 290 °C was used and the initial column temperature was set at 100 °C, which was held for 3 min followed by a 10 °C/min ramp to 180 °C, then a 6 °C /min ramp to 300 °C which was held for 2 min (total run time = 33 min). Interface and ion source temperatures were 260 °C and 250 °C respectively. The HRMS was operated in positive electron-impact ionisation mode (+EI) with an electron energy of 35 eV and at a resolution of 10,000. The eV of 35 versus 70, which is traditionally employed in +EI, was chosen as it resulted in reduced fragmentation of the parent ions. Selected ion monitoring was used to detect the trimethylsilylated derivatives of the target analyte compounds.

APPENDIX II – GC-HRM methodology details (Continued)

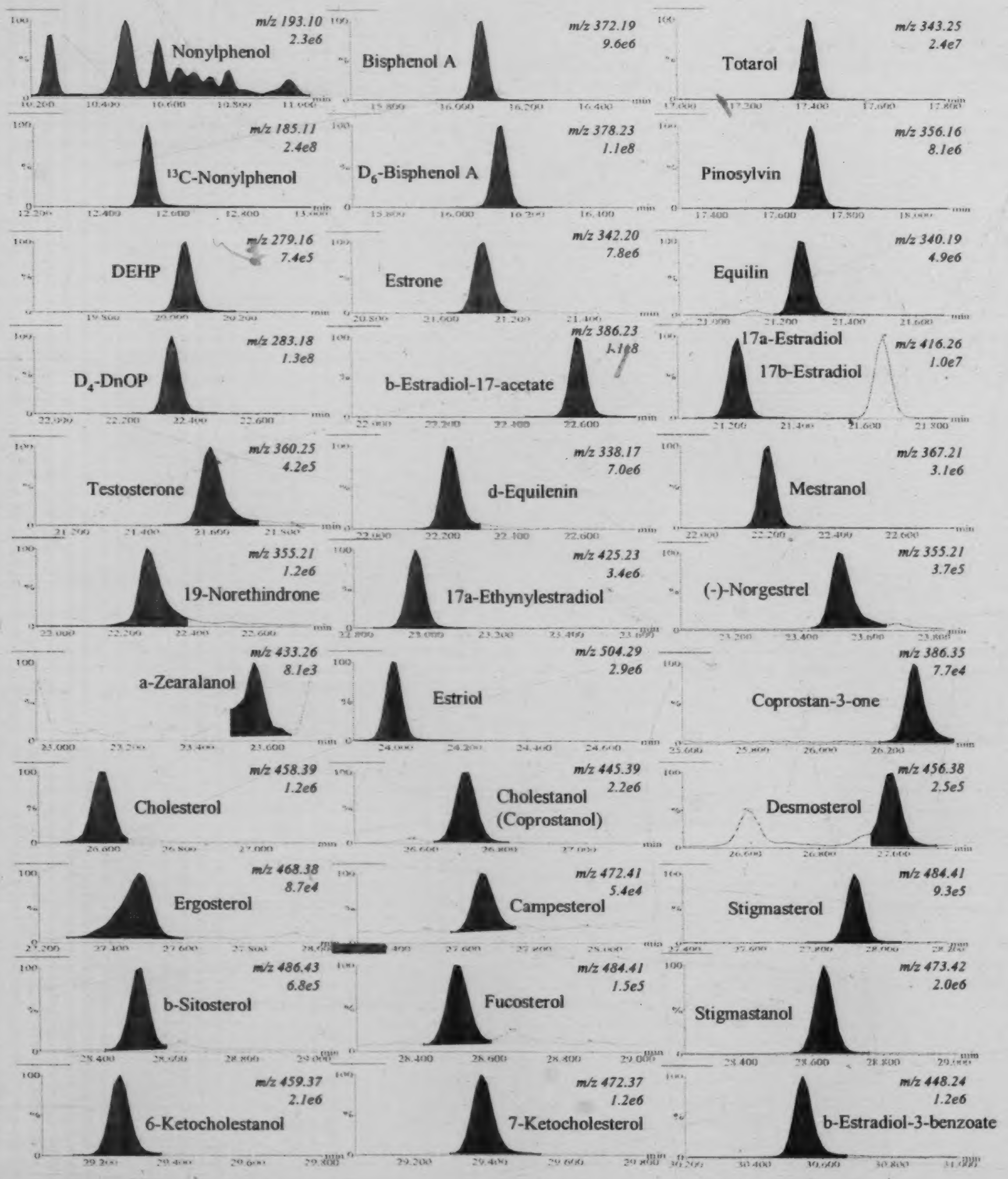

Figure A2-1. Selected Ion Monitoring (SIM) with accurate *m/z* for each compound chromatograms for each analyte and IS monitored.

APPENDIX II – GC-HRM methodology details (Continued)

QA/QC

The criteria for analyte quantitation and quality assurance were adapted from an in-house protocol for ultra-trace organohalogen analysis by GC-HRMS[1] Two isotopes of a specific analyte must be detected at their exact m/z and at 10,000 resolution. Both of the isotope signals must be present, and must maximize within ±2 s of one another. The retention time of a specific analyte must be within 3 s to that obtained during analysis of the authentic compounds in the calibration standards. The signal-to-noise ratio of each of the monitored ions must be ≥3 and the ratio of the two isotopic peaks must be 20% of the theoretical value otherwise it was reported as not detected due to incorrect isotopic ratio. Samples were processed in batches of 12 that contained nine samples, one duplicate, one blank, and one spiked sample (50 µl of ASM). A six-point calibration curve was prepared using 1, 2, 5, 10, 20, and 50 µl of the ASM with 50 µl of internal standard mixture in each. This mixture was reduced under nitrogen, derivatized as described for the samples and was run on the GC-HRMS at the beginning of each batch of 12 to 24 samples. The mid-range standards were periodically reanalyzed throughout the batch to ensure no drifts in calibration occurred during the analysis period. Quadratic calibration curves were use to model the internal standard normalized response of each analyte to provide a better fit (compared to a linear function) and more accurate quantitation for each compound. Quadratic coefficients were determined for each batch of processed samples using quadratic curvi-linear regression in SPSS® 10.0 (SPSS, Chicago, IL, USA) for the six-point calibration data along with any other calibration standards (i.e., mid-range standards) run throughout the batch. Quantitation limits were established based on the limits of quantitation as defined by the calibration curves. If the response of any analyte exceeded the range defined by the calibration curves a 1:6 dilution with DCM was carried out and the sample was reanalyzed to put the response in the quantifiable range. Method detection limits (MDLs) were based on signal to noise ratio (S/N) of approximately 3:1 for the ion with the lowest S/N ratio of the two used to monitor each analyte. Noise was determined as ±2 standard deviations determined for a representative sample of the baseline nearest to the peak in a spiked wastewater sample.

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[1] Ikonomou MG, Fraser TL, Crewe NF, Fischer MB, Rogers IH, He T, Sather PJ, Lamb RF. 2001. A comprehensive multi-residue ultra-trace analytical method , based on HRGC/HRMS, for the determination of PCDDs, PCDFs, PCBs, PBDEs, PCDEs, and organohalogen pesticides in six different environmental matrices. *Can Data Rep Fish Aquat Sci* 2389:1-95.

APPENDIX III – RYA protocol and data analysis

The recombinant yeast strain used in this was modified to contain the DNA sequence of the human estrogen receptor (hER) on the main chromosome as well as an expression plasmid carrying the reporter gene *Lac-Z* which encodes for the enzyme β-galactosidase. The hER is expressed in a form capable of binding to estrogen-reponseive elements (ERE) on the plasmid and once activated by any true estrogen causes expression of the reporter gene which produces β-galactosidase. The amount of β-galactosidase produced was then monitored colorometrically by assaying with chlorophenol red-β-D-galactopyranoside (CPRG) which is normally yellow but produces a red product with an absorbance maxima at 575 nm upon cleavage of the β-glycosidic bond. The following modifications were implemented: PCR sealing film (Axygen Scientific, Union City, CA, USA) was used to seal the plates instead of autoclave tape prior to incubation, and the wells were agitated using a vortex stirrer fitted with a 96-well plate adaptor prior to reading on the third day of incubation. The 96-well plates were Becton-Dickinson (Frankilin Lakes, NY, USA) Microtest™ tissue culture flat bottom plates. Additionally, we used a Boekel Scientific (Pennsylvania, USA) naturally ventilated incubator and 8-channel (40-200 μL) Nichipet® 7000 and single channel Nichipet® Ex (10-100 μL) micro pipettors (Nichiryo, Tokyo, Japan).

17β-estradiol (E2; ≥98 % cell culture tested; Sigma-Aldrich, Oakville, ON, Canada) standards were prepared in anhydrous ethanol at well concentrations (10 μL per well made up to 200 μL of assay medium) of 0.76 ng/L, 1.93 ng/L, 3.85 ng/L, 7.70 ng/L, 13.5 ng/L, 27.0 ng/L, 48.8 ng/L, 86.3 ng/L, 189 ng/L, 323 ng/L, 755 ng/L, 1509 ng/L. Absorbance at 540 nm and optical density at 650 nm were determined on a Molecular Devices (Sunnyvale, CA, USA) Vmax® microplate reader for each well after the incubation period. For calculation of β-galactosidase activity, we used the ratio of 540 nm to 650 nm (optical density) minus the same ratio from an appropriate blank well. Dose-response curves (DRCs) were plotted as the blank subtracted β-galactosidase activity for all twelve E2 standards run in triplicate verses the log of the concentration in grams per liter. The curves were fit using a signoidal dose-response curve (variable slope), Marquardt-Levenberg algorithm in SigmaPlot version 8.0 (SPSS, Chicago, IL, USA). Coefficients of determination (r^2) of 0.998 were observed for our dose-response curves. RYA detection limits were 4-8 ng/L E2-equivalents based on a 200 mL sample SPE extracted, eluted into a 5 mL fraction taken to dryness under nitrogen, resuspended in 300 uL, and finally, a 10 uL aliquot applied to a 200 uLs of medium in a 96-well plate. Reproducibility was found to be <10% for triplicate standard doses of E2 run above detection limit.

Calculation of E2-equivalents (X) was performed using the modeled does-response equation (1) and the measured blank subtracted β-galactosidase activity (Y) for the unknown sample.

$$Y = \min + \left(\frac{\max - \min}{1 + 10^{\left[\log EC50 - X\right] hillslope}} \right) \qquad (1)$$

Where min = minimum of DRC; max = maximum of DRC; EC50 = concentration of agonist at 50% maximal response; hillslope = slope of linear portion of DRC.

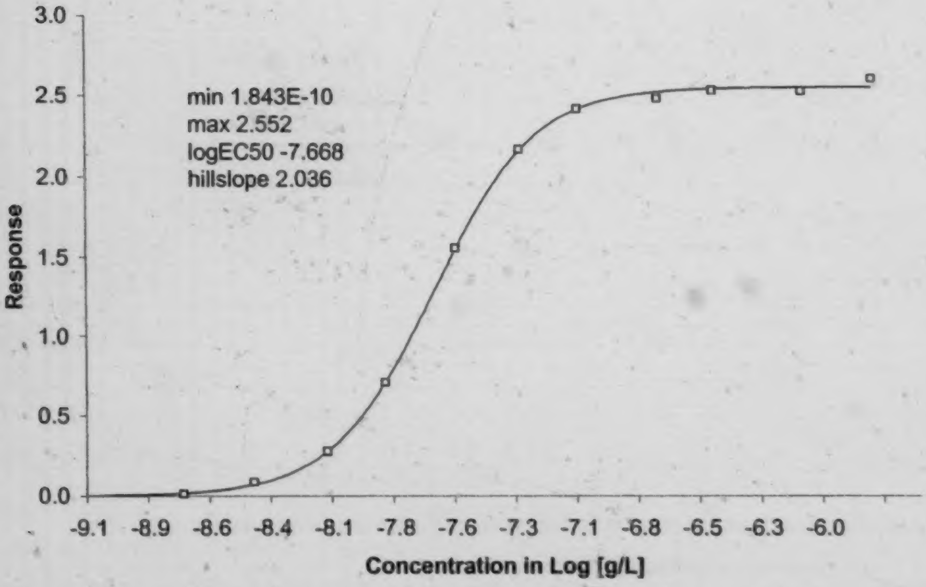


Figure A2-2. Typical DRC obtained for RYA (each point represents a mean of 3 replicates).

APPENDIX IV - Chemical and *in vitro* data for all samples analyzed in this work.

Compounds (ng/L)	Nonylphenol	Bisphenol A	Totarol	Pinosylvin	DEHP	Estrone	Equilin	17a-Estradiol	Testosterone	17b-Estradiol
Site A										
Eff1	n/a	11	148	0	708	0	0	10	0	0
Eff2	n/a	40	127	23	937	0	0	12	0	0
Eff3	n/a	26	111	9	1093	0	0	17	0	0
Eff4	n/a	19	99	6	942	0	0	0	0	12
Eff5	n/a	13	152	2	680	0	0	0	0	0
Eff6	n/a	16	288	3	948	49	0	0	0	4
Ave	n/a	21	154	7	885	8	0	6	0	3
Site B										
Inf1	19278	418	0	3	11165	0	0	0	0	0
Eff1	10039	353	0	52	4676	0	0	2*	0	5
Inf2	24605	350	0	1	12230	17	0	1*	95	11
Eff2	5708	208	1	0	2052	69	0	0	16*	2
Inf3	18842	190	13	0	12375	12	0	1*	0	8
Eff3	10129	152	0	0	1555	147	0	1*	0	10
Inf4	15385	195	0	0	10936	8	0	0	64	10
Eff4	9554	112	0	0	2301	0	0	0	0	1
Inf5	14728	88	6	0	11165	0	0	0	0	2
Eff5	10776	77	2	1	3962	0	0	1*	21*	3
Inf6	40850	314	199	0	12240	9	0	0	0	0
Eff6	12854	220	0	0	2610	0	0	0	0	6
Inf7	32400	281	208	0	11628	4*	0	0	0	1*
Eff7	12454	153	92	3	2080	25	0	0	0	6
Inf8	41207	438	220	0	12949	19	0	0	0	8
Eff8	11349	351	0	1	2483	85	0	1*	0	12
Ave(inf)	25912	284	81	1	11836	9	0	0	20	5
Ave(eff)	10358	203	12	7	2715	41	0	1	5	5
%Difference	60%	28%	85%	-1308%	77%	-370%	0%	-171%	76%	-11%

NOTE: The rows showing "%Difference" are estimated from the influent / effluent samples taken during the various sampling campaigns. No statistical analyses were performed on these values as they only serve as estimates due to the fact that hydraulically matched flow proportional sampling was not available at each plant. *Value below method detection limit.

APPENDIX IV (Continued)

Compounds (ng/L)	Nonylphenol	Bisphenol A	Totarol	Pinosylvin	DEHP	Estrone	Equilin	17a-Estradiol	Testosterone	17b-Estradiol
Site B										
Eff1a	7854	420	183	0	5347	2*	0	0	0	3
Eff1b	10037	431	183	0	3941	0	0	0	0	0
Eff1c	14836	610	0	0	2032	1*	0	0	0	0
Eff1d	22033	719	0	0	2705	1*	0	0	0	0
Eff2a	14974	59	136	11	1933	45	207	37	0	23
Eff2b	16804	210	138	11	1863	41	0	37	0	25
Eff2c	14688	258	137	0	2343	21	0	0	0	10
Eff2d	17567	202	137	0	1784	40	0	0	0	23
Eff3a	12510	859	137	11	1512	44	0	0	0	25
Eff3b	18440	1054	137	11	647	54	0	0	0	25
Eff3c	12022	953	137	11	552	52	0	38	0	25
Eff3d	90043	464	138	11	5770	54	0	0	0	25
Eff4a	20700	500	137	11	3063	40	0	37	0	25
Eff4b	19605	647	144	0	2865	0	0	0	0	3
Eff4c	18509	701	143	0	2639	0	0	0	0	5
Eff4d	19415	759	145	0	3645	0	0	0	0	6
Ave(eff)	20627	553	127	5	2665	25	13	9	0	14
Site C										
Inf1	28207	186	0	14	3471	33	0	0	46	0
Eff1	4136	33	0	0	869	10	0	0	0	0
%Difference	85%	82%	0%	100%	75%	70%	0%	0%	100%	0%
Site D										
Inf1	15427	590	0	2	9960	9	0	0	42	1*
Eff1	1592	0	0	0	17092	18	0	0	0	0
%Difference	90%	100%	0%	100%	-72%	-100%	0%	0%	100%	100%
Site E										
Eff1	4466	108	0	0	4089	28	0	0	0	57
Eff2	5009	119	0	0	5091	39	0	0	0	72
Inf3	2553	0	0	0	5217	0	0	0	0	7
Eff3	2803	88	0	8	3704	56	83	3*	0	158
%Difference	-10%	"created"	0%	"created"	29%	"created"	"created"	"created"	0%	-2034%
Ave%Reduction	56%	70%	21%	-369%	27%	-133%	0%	-57%	69%	-486%

APPENDIX IV (Continued)

Compounds (ng/L)	d-Equilenin	Mestranol	19-Norethindrone	17a-Ethynylestradiol	(-)-Norgestrel	a-Zearalanol	Estriol	Coprostan-3-one
Site A								
Eff1	0	234	0	0	0	0	0	0
Eff2	0	0	0	0	0	0	0	0
Eff3	0	0	0	0	0	0	0	140
Eff4	0	0	0	0	0	0	0	0
Eff5	0	0	0	0	0	0	0	113
Eff6	0	0	0	0	0	0	0	0
Ave	0	39	0	0	0	0	0	42
Site B								
Inf1	0	0	0	0	0	0	5	160477
Eff1	0	0	0	1*	0	0	8	17611
Inf2	0	0	0	2*	0	0	7	230119
Eff2	0	0	159	0	0	0	0	18132
Inf3	0	0	87	2*	48*	0	7	225734
Eff3	0	0	0	1*	0	0	0	20020
Inf4	0	0	0	2*	0	0	20	190096
Eff4	0	156	0	0	0	0	0	14130
Inf5	0	0	0	0	0	0	3	161996
Eff5	0	0	0	1*	93	0	0	23240
Inf6	0	0	213	0	0	0	5	121778
Eff6	0	0	0	0	0	0	0	11139
Inf7	0	0	213	0	0	0	2	94889
Eff7	9	0	0	0	0	0	1*	5686
Inf8	0	0	224	0	0	0	22	97890
Eff8	3*	0	0	131	0	0	0	8223
Ave(inf)	0	0	92	1	6	0	9	160372
Ave(eff)	1	19	20	17	12	0	1	14773
%Difference	"created"	"created"	78%	-2096%	-93%	0%	87%	91%

APPENDIX IV (Continued)

Compounds (ng/L)	d-Equilenin	Mestranol	19-Norethindrone	17a-Ethynylestradiol	(-)-Norgestrel	a-Zearalanol	Estriol	Coprostan-3-one
Eff1a	1*	0	0	0	0	0	0	9286
Eff1b	0	0	0	0	0	0	0	8946
Eff1c	0	0	0	0	0	0	0	6515
Eff1d	0	0	0	0	0	0	0	8361
Eff2a	0	0	0	0	0	0	29	8647
Eff2b	0	0	0	0	125	0	29	6998
Eff2c	0	0	0	0	126	0	14	12732
Eff2d	0	0	0	0	124	0	29	9454
Eff3a	0	0	0	174	0	0	29	10069
Eff3b	0	0	0	0	0	0	29	4546
Eff3c	0	0	0	0	0	0	29	8232
Eff3d	0	0	0	175	0	0	29	11861
Eff4a	0	0	0	0	0	0	28	6756
Eff4b	0	0	0	178	0	0	0	6925
Eff4c	0	0	0	178	0	0	0	6517
Eff4d	0	0	0	178	0	0	0	7403
Ave(eff)	0	0	0	55	23	0	15	8328
Site C								
Inf1	0	0	26	0	0	0	0	93071
Eff1	0	0	0	0	0	0	0	8746
%Difference	0%	0%	100%	0%	0%	0%	0%	91%
Site D								
Inf1	0	0	26*	0	0	20	0	2448
Eff1	0	0	0	0	0	0	0	3866
%Difference	0%	0%	100%	0%	0%	100%	0%	-58%
Site E								
Eff1	1*	0	0	0	0	0	0	69704
Eff2	13	0	0	0	0	0	4	58416
Inf3	1*	0	0	0	0	0	0	161187
Eff3	11	0	0	5*	0	0	23	55334
%Difference	-1530%	0%	0%	"created"	0%	0%	"created"	66%
Ave%Difference	-510%	0%	70%	-699%	-23%	25%	29%	47%

APPENDIX IV (Continued)

Compounds (ng/L)	Cholesterol	Coprostanol	Desmosterol	Ergosterol	Campesterol	Stigmasterol	b-Sitosterol	Fucosterol	Stigmastanol
Site A									
Eff1	452	179	0	636	2299	789	6679	12136	4035
Eff2	520	161	0	764	1445	335	4825	7823	2430
Eff3	3404	166	0	1460	3553	536	11812	18918	7129
Eff4	1665	161	0	9124	1982	332	5799	9467	3081
Eff5	422	151	0	2593	2866	582	8072	12013	5470
Eff6	196	153	0	452	2407	166	6058	10537	3669
Ave	1110	162	0	2505	2425	457	7207	11816	4302
Site B									
Inf1	70486	6080	3083	524	6936	7084	27113	36647	8631
Eff1	11560	1656	492	0	1232	805	350	2741	697
Inf2	90237	8401	4857	810	8563	13860	32695	41611	7116
Eff2	12583	1478	633	343	1378	3000	5837	9291	667
Inf3	80478	7199	3775	808	7980	10331	27160	36562	6900
Eff3	13729	1694	580	0	1575	4104	3169	7531	588
Inf4	78929	7234	3449	0	7513	8952	27020	37554	7849
Eff4	12600	1797	712	0	1536	2757	5631	9707	762
Inf5	90869	7749	4187	1550	7999	8576	26981	37214	7252
Eff5	13176	2191	744	703	1574	2972	4706	8746	787
Inf6	112302	8399	4000	4665	8812	12239	35033	48916	11728
Eff6	17701	2628	698	1383	1907	7206	10719	15355	1242
Inf7	88257	6501	3806	3740	8123	8677	30486	41758	9365
Eff7	10905	1461	643	2090	1355	2641	6617	8708	713
Inf8	99121	7146	3390	3840	7750	10242	30134	39952	9740
Eff8	13935	2013	912	0	1646	3700	6641	9833	938
Ave(inf)	88835	7339	3818	1992	7959	9995	29578	40027	8573
Ave(eff)	13274	1865	677	565	1525	3398	5459	8989	799
%Difference	85%	75%	82%	72%	81%	66%	82%	78%	91%

APPENDIX IV (Continued)

Compounds (ng/L)	Cholesterol	Coprostanol	Desmosterol	Ergosterol	Campesterol	Stigmasterol	b-Sitosterol	Fucosterol	Stigmastanol
Eff1a	14998	2467	1231	1824	2239	2531	2901	8193	896
Eff1b	11371	2279	919	1354	1800	2489	3014	8540	787
Eff1c	9816	1851	808	2602	1511	2214	5056	8517	726
Eff1d	10864	2041	776	1247	1469	1211	1876	5420	740
Eff2a	13292	1967	857	1445	1655	2490	4361	7297	770
Eff2b	19120	2480	1042	1315	1977	2129	3005	7069	950
Eff2c	18431	2625	1024	1798	2185	3077	7370	12140	1252
Eff2d	14687	2301	943	1352	1836	2093	3345	6973	884
Eff3a	14111	2226	951	506	1615	3027	5659	9660	1021
Eff3b	8694	1433	633	588	1360	5480	12771	15340	855
Eff3c	12006	1799	734	730	1515	2866	7289	10469	906
Eff3d	20163	1878	1459	1505	3420	27100	42110	33599	1696
Eff4a	9871	1678	822	1591	1291	4343	9464	13016	751
Eff4b	8548	1384	540	566	1120	3480	6391	10348	619
Eff4c	11260	1670	693	640	1266	3147	5595	10230	750
Eff4d	11077	1652	775	747	1429	5126	10977	15120	962
Ave(eff)	13019	1983	888	1238	1731	4550	8199	11371	910
Site C									
Inf1	18418	3235	3598	841	1655	2729	7303	9470	3201
Eff1	2686	517	269	284	316	689	1366	2008	244
%Difference	85%	84%	93%	66%	81%	75%	81%	79%	92%
Site D									
Inf1	42146	1849	4243	1118	530	403	16123	13306	1141
Eff1	2601	282	144	312	268	1588	929	1734	96
%Difference	94%	85%	97%	72%	49%	-294%	94%	87%	92%
Site E									
Eff1	35754	2236	0	399	5360	15527	17914	47597	1415
Eff2	38628	2622	0	0	5049	20434	10447	43485	1307
Inf3	136935	4811	0	2044	12119	6140	33635	51029	7083
Eff3	33359	2697	0	0	4418	17491	8793	32299	1182
%Difference	76%	44%	0%	100%	64%	-185%	74%	37%	83%
Ave%Difference	85%	72%	68%	77%	69%	-84%	83%	70%	89%

APPENDIX IV (Continued)

Compounds (ng/L)	6-Ketocholestanol	7-Ketocholesterol	b-Estradiol-3-benzoate	Chemical EEq (ng/L)	RYA-Whole	RYA - Fr1	RYA - Fr2	RYA - Fr3	RYA - Fr4	RYA - Fr5
Site A										
Eff1	0	25	0	1	48	n/a	n/a	n/a	n/a	n/a
Eff2	4*	42	0	1	61	2	3	6	3	2
Eff3	4*	76	0	1	66	2	2	11	3	1
Eff4	0	46	0	12	98	n/a	n/a	n/a	n/a	n/a
Eff5	2*	0	0	0	62	2	2	5	1	1
Eff6	7	0	0	9	40	2	2	9	2	0
Ave	3	31	0	4	63	2	2	8	2	1
Site B										
Inf1	368	3522	0	1	76	1	3	20	5	2
Eff1	18	1052	0	7	66	2	0	18	7	2
Inf2	424	6259	0	16	106	2	2	43	4	2
Eff2	17	665	2	9	72	0	0	25	16	2
Inf3	308	4182	5	12	99	n/a	n/a	n/a	n/a	n/a
Eff3	14	238	0	26	80	n/a	n/a	n/a	n/a	n/a
Inf4	306	4650	0	13	86	2	2	46	5	1
Eff4	17	225	0	1	61	0	0	24	13	2
Inf5	233	4321	0	2	86	2	2	96	6	1
Eff5	24	484	5	4	56	1	1	16	9	0
Inf6	927	7707	0	3	85	n/a	n/a	n/a	n/a	n/a
Eff6	30	800	0	6	77	n/a	n/a	n/a	n/a	n/a
Inf7	766	6932	0	3	89	2	2	39	4	0
Eff7	36	1615	49	9	69	2	1	33	13	0
Inf8	689	7239	0	12	90	2	2	81	5	0
Eff8	46	2158	0	136	57	1	2	20	9	1
Ave(inf)	503	5601	1	8	89	2	2	54	5	1
Ave(eff)	25	905	7	25	67	1	1	23	11	1
%Difference	95%	84%	-1004%	-216%	25%	49%	68%	58%	-126%	-21%

APPENDIX IV (Continued)

Compounds (ng/L)	6-Ketocholestanol	7-Ketocholesterol	b-Estradiol-3-benzoate	Chemical EEq (ng/L)	RYA-Whole	RYA - Fr1	RYA - Fr2	RYA - Fr3	RYA - Fr4	RYA - Fr5
Eff1a	58	1931	0	4	N/A	0	2	5	2	2
Eff1b	50	1826	0	1	N/A	0	0	3	1	0
Eff1c	35	1306	0	1	N/A	0	0	2	0	0
Eff1d	43	1412	0	1	N/A	0	0	2	0	0
Eff2a	38	439	0	31	N/A	2	2	7	0	0
Eff2b	42	148	0	32	N/A	0	0	6	0	0
Eff2c	38	325	0	13	N/A	0	0	5	0	0
Eff2d	41	343	0	28	N/A	0	0	9	2	0
Eff3a	45	407	0	185	N/A	0	0	3	0	0
Eff3b	32	143	0	32	N/A	0	0	5	1	0
Eff3c	39	245	0	33	N/A	0	0	1	0	0
Eff3d	64	687	0	191	N/A	0	0	5	0	0
Eff4a	39	374	0	32	N/A	0	0	5	1	0
Eff4b	12	248	0	162	N/A	0	0	3	0	0
Eff4c	12	226	0	164	N/A	0	0	3	0	0
Eff4d	16	235	0	165	N/A	0	0	2	0	0
Ave(eff)	38	643	0	67	N/A	0	0	4	0	0
Site C										
Inf1	173	1010	0	5	N/A	0	0	26	5	0
Eff1	0	18	0	1	N/A	4	0	7	4	0
%Difference	100%	98%	0%	75%	N/A	"created"	0%	72%	10%	0%
Site D										
Inf1	279	2451	0	3	N/A	7	4	33	9	0
Eff1	0	15	0	2	N/A	3	3	10	4	0
%Difference	100%	99%	0%	42%	N/A	57%	29%	69%	56%	0%
Site E										
Eff1	47	404	0	59	9	1	1	8	3	0
Eff2	51	349	0	76	11	0	0	6	0	0
Inf3	179	1455	0	8	54	1	2	1	0	0
Eff3	41	360	0	168	30	2	2	6	2	0
%Difference	77%	75%	0%	-2130%	43%	-218%	9%	-799%	-526%	0%
Ave%Difference	93%	89%	-251%	-557%	34%	-38%	26%	-150%	-146%	-5%

APPENDIX V - MDLs average and ranges based on signal-to-noise of 3 using spiked wastewater (n=3) via GC-HRMS.

Compound	**MDL in ng/L**	**Compound**	**MDL in ng/L**
Nonylphenol	172(115-219)	α-Zearalanol	529(246-903)
Bisphenol A	2.1(1.7-2.4)	Estriol	1.5(1.1-2.3)
Totarol	1.0(0.5-1.8)	Coprostan-3-one	28 (17-49)
Pinosylvin	14(1.8-27)	Cholesterol	25(5.8-44)
DEHP	20(13-25)	Cholestanol	22(2.5-35)
Estrone	7.6(5.0-11)	Desmosterol	83(52-135)
Equilin	18(9.3-28)	Ergosterol	139(65-213)
17α-Estradiol	6.9(4.5-11)	Campesterol	11(6.8-19)
Testosterone	33(22-41)	Stigmasterol	18(4.1-31)
17β-Estradiol	7.1(1.6-12)	β-Sitosterol	23(12-39)
d-Equilenin	17(4.1-31)	Fucosterol	260(200-310)
Mestranol	19(6.4-28)	Stigmastanol	24(10-49)
19-Norethindrone	38(32-45)	6-Ketocholestanol	6.1(4.6-7.9)
17α-Ethynylestradiol	7.1(6.1-9.0)	7-Ketocholesterol	23(5.7-51)
(-)-Norgestrel	84(74-98)	17β-Estradiol-3-benzoate	6.6(3.8-12)

APPENDIX VI – Summary of SPE spike experiment (average of n=3 for each fraction)

The SPE fractionation procedure was validated for the recovery of potential EDC compounds using a mixture of labelled surrogate standards spiked into wastewater samples. 100 µL of ISM (see Table A2-1 in Appendix II; however β-Estradiol-C^{13} was used instead of β-Estradiol-17-acetate due to availability issues), were added to 200 mL of AIWWTP 2° effluent in triplicate and this solution was extracted via SPE as discussed previously. The elution sequence was 5mL each of 25% MeOH, 50% MeOH, 100% MeOH, diethyl ether and cylcohexane for a total of 2 aqueous fractions and 3 organic fractions. Table X shows the recoveries for the latter 3 organic fractions. As our determination method is based on GC-HRMS following derivatization, the great difficulty and variability introduced in preparing the aqueous fractions (i.e. 25% MeOH and 50% MeOH) coupled with the fact that our target compounds are classified as mid-polar to non-polar we only analyzed the organic fractions for recovery of the labelled compounds.

Compound	Fr III (MeOH)	Fr IV (Et_2O)	Fr V (Cyclohexane)	Total
Ring-$^{13}C_6$-nonylphenol	9	23	2	35
Propane-d_6-bisphenol A	67	8	0	74
Di-n-octylphthalate-d_4	3	25	0	28
β-Estradiol-^{13}C	54	5	0	59
2,2,3,4,4,6-d_6-cholesterol	7	29	0	36

All values represent %recovery.

APPENDIX VII – RYA Response (Abs 570 nm/Abs 620 nm) to BPA addition in wastewater Fraction 3 for three different wastewater effluent samples surveyed in this work.

BPA Added (μg/L)	*Gold Bar Effluent*	*SD*	*AIWWTP2° 27/03/03 3PM*	*SD*	*AIWWTP2° 03/04/03 3AM*	*SD*
0 (control)	2.61	0.36	1.50	0.04	3.27	0.22
0.5	2.42	0.38	1.47	0.22	2.89	0.20
1.0	2.23	0.33	1.38	0.04	3.08	0.19
1.8	2.37	0.32	1.49	0.10	3.05	0.23

APPENDIX VIII – Information on internal standards and analytes measured via LC-MS/MS

Table A4-1 - Internal standard mixture (ISM) amounts in each 50uL of IS Mix and MS/MS ions monitored.

Compound	*Amount (ng)*	*Ion*	*Molecular ion m/z*	*Product Ions m/z*
Estrone-2,4,16-d_4--3-Sulfate	30	Neg	353.3	80.0, 273.0[b]
3,4-$^{13}C_2$-Estradiol	12	Neg	273.0	147.2, 184.8[b]
^{13}C-Nonylphenol[a]	19	Neg	225.5	111.9
Propane-d_6-Bisphenol A	25	Neg	233.4	138.0, 214.2
2,2,3,4,4,6-d_6-Cholesterol[a]	494	na	Analyzed via GC-HRMS	na

[a] Monitored via previously published GC-HRMS method as cited in text; [b] Ion(s) used for quantitation.

Table A4-2 - Analytes, corresponding internal standards used for quantitation, ionization mode, and MS/MS analyte ions monitored.

Compound	*IS*	*Ion*	*Parent m/z*	*Daughters m/z*
Estrone-3-sulphate	Estrone-2,4,16-d_4--3-Sulfate	Neg	349.3	269.1[b], 145.0
Estrone-3-glucuronide	Estrone-2,4,16-d_4--3-Sulfate	Neg	445.3	269.3[b], 113.0[b]
Estradiol-3-sulphate	Estrone-2,4,16-d_4--3-Sulfate	Neg	351.4	271.0[b], 80.0
Estradiol-3-glucuronide	Estrone-2,4,16-d_4--3-Sulfate	Neg	447.5	271.0[b], 113.0
Estriol-3-sulphate	Estrone-2,4,16-d_4--3-Sulfate	Neg	367.3	287.0[b], 80.0
Estradiol-16-glucuronide	Estrone-2,4,16-d_4--3-Sulfate	Neg	463.0	287.0[b], 113.1, 85.0
Ethinylestradiol-3-sulphate	Estrone-2,4,16-d_4--3-Sulfate	Neg	375.5	295.1[b], 79.9
Ethinylestradiol-3-glucuronide	Estrone-2,4,16-d_4--3-Sulfate	Neg	471.5	295.2[b], 113.0
17α/β-Estradiol	3,4-$^{13}C_2$-Estradiol	Neg	271.4	120.2, 146.1[b], 183.9
Estrone	3,4-$^{13}C_2$-Estradiol	Neg	269.1	145.0[b], 159.2
Estriol	3,4-$^{13}C_2$-Estradiol	Neg	287.2	171.1[b], 183.1
16α-hydroxyestrone	3,4-$^{13}C_2$-Estradiol	Neg	285.2	159.0, 145.0[b]
17α-Ethinylestradiol	3,4-$^{13}C_2$-Estradiol	Neg	295.2	144.9[b], 159.1, 199.2
19-Norethindrone	3,4-$^{13}C_2$-Estradiol	Pos	299.1	130.9[b], 104.9
(-)-Norgestrel	3,4-$^{13}C_2$-Estradiol	Pos	313.2	245.1[b], 295.0
Diethylstilbesterol	3,4-$^{13}C_2$-Estradiol	Neg	267.2	222.0[b], 237.0
Progesterone	3,4-$^{13}C_2$-Estradiol	Pos	315.2	109.1[b], 297.2
Equilenin	3,4-$^{13}C_2$-Estradiol	Neg	265.2	248.9, 221.0[b]
Equilin	3,4-$^{13}C_2$-Estradiol	Neg	267.1	115.1, 143.0[b]
α-Zearalanol	3,4-$^{13}C_2$-Estradiol	Neg	321.2	161.2[b], 277.0
17β-Estradiol-3-benzoate	3,4-$^{13}C_2$-Estradiol	Pos	377.1	77.1[b], 105.1
Testosterone	3,4-$^{13}C_2$-Estradiol	Neg	289.2	96.9, 109.1[b]
Bisphenol A	Propane-d_6-Bisphenol A[a]	Neg	227.4	133.0[b], 210.9

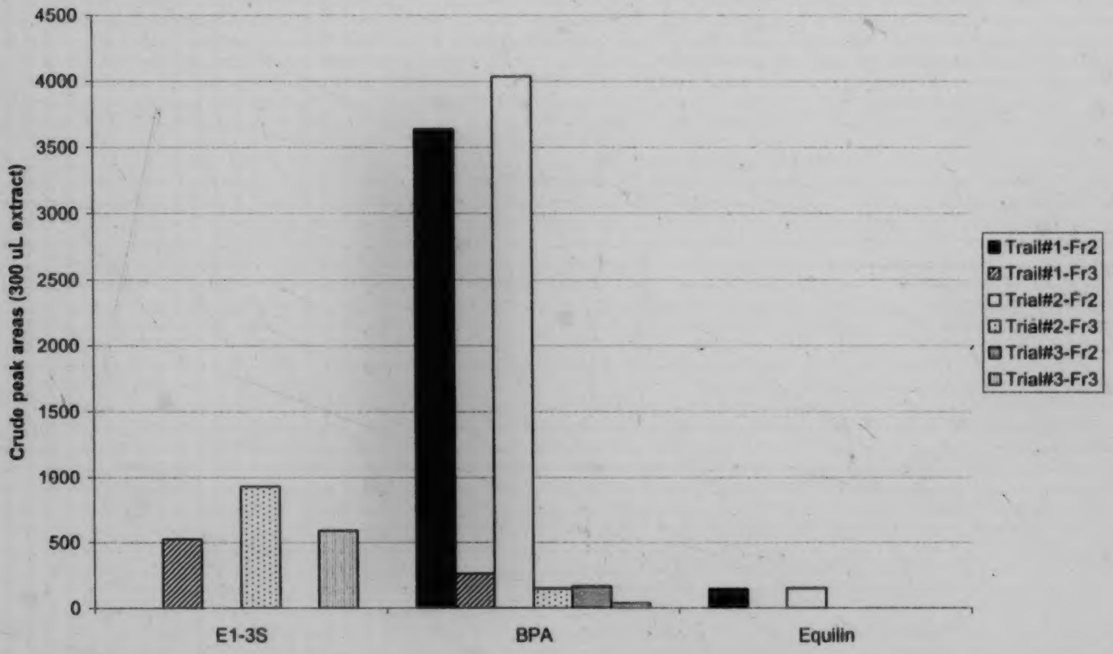
[a] Internal standard was not detected via LC-MS/MS in samples thus, external calibration was used; [b] Ion(s) used for quantitation.

APPENDIX IX – SPE method development trials.

Fifty microliters of IS solution (0.61 ng/µL BPA-d_4, 0.082 ng/µL Equilin-d_4, 0.080 ng/µL E1-3S-d_4) was added to 100 mL of wastewater effluent for the following SPE elution solvent method development trials.

Trial#1 = Fr1 - diethyl ether; Fr2 - ethyl acetate; Fr3 - methanol with 5 mM TEA
Trail#2 = Fr1 - diethyl ether; Fr2 - diethyl acetate; Fr3 – 80% methanol
Trial#3 = Fr1 - diethyl ether/10% isopropynol; Fr2 - ethyl acetate; Fr3 – methanol

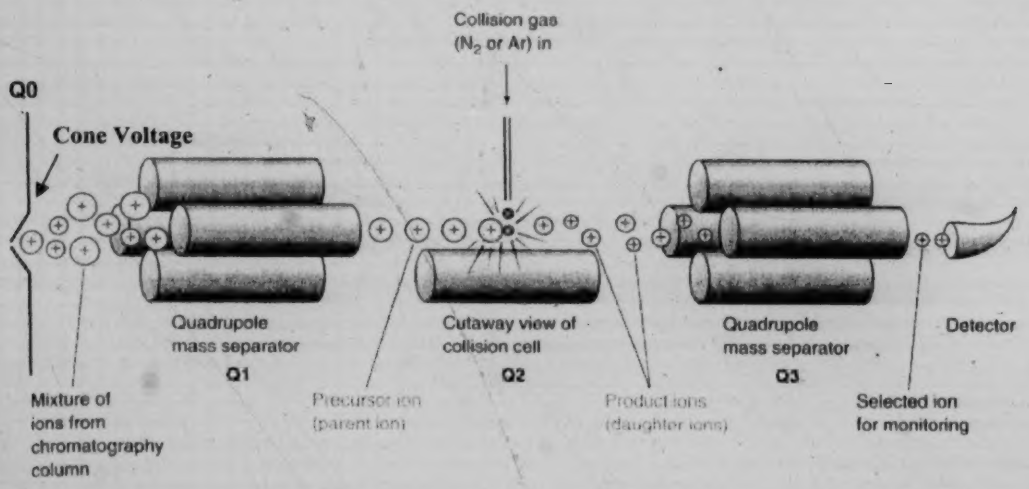
Compounds were detected via negative ion electorspray ionization LC -MS/MS analysis. The following transitions were monitored of each analyte: BPA-d_4 = 231.0 > 216, 215, 135 *m/z*; Equilin-d_4 = 271 > 269, 145 *m/z*; E1-3S-d_4 = 353.0 > 274, 147.



APPENDIX X – LC gradient conditions used for liquid chromatographic gradients 1&2

LC Method 1 (Conjugated estrogens)			*LC Method 2 (Free estrogens)*		
Time (min)	% Acetonitrile	% Water	Time (min)	% Methanol	% Water
0	10	90	0	10	90
1	10	90	1	10	90
11	40	60	20	95	5
14	90	10	20.1	10	90
17	90	10	25	10	90
17.1	10	90			
20	10	90			

APPENDIX XI – Illustrations of the components of a typical MS/MS unit


Collision gas
(N_2 or Ar) in
Q0
Cone Voltage
Quadrupole
mass separator
Q1
Cutaway view of
collision cell
Q2
Quadrupole
mass separator
Q3
Detector
Mixture of
ions from
chromatography
column
Precursor ion
(parent ion)
Product ions
(daughter ions)
Selected ion
for monitoring

Adapted from Harris DC. 2007. Quantitative chemical analysis. New York: W.H. Freeman & Company.

APPENDIX XII – Monte Carlo analysis for E1 secondary K_d error estimate.

E1	**Log $C_{w,floc}$** *1.886*±4.93%	**10^**	**Log $C_{s,floc}$** *0.991*±4.93%	**10^**	**Kd**
Trial#					
1	1.933	85.7	1.029	10.7	0.125
2	2.003	100.8	1.075	11.9	0.118
3	1.824	66.6	0.969	9.3	0.140
4	1.788	61.3	0.990	9.8	0.159
5	1.904	80.2	1.037	10.9	0.136
6	1.900	79.5	1.091	12.3	0.155
7	1.860	72.4	0.988	9.7	0.135
8	1.807	64.0	0.954	9.0	0.140
9	1.897	78.9	0.985	9.7	0.122
10	1.948	88.7	0.979	9.5	0.107
11	1.776	59.7	0.924	8.4	0.141
12	1.854	71.5	0.998	10.0	0.139
13	1.825	66.8	1.011	10.3	0.154
14	1.772	59.1	0.888	7.7	0.131
15	1.811	64.7	1.030	10.7	0.166
16	1.937	86.5	1.083	12.1	0.140
17	2.021	105.0	0.970	9.3	0.089
18	1.821	66.2	1.038	10.9	0.165
19	1.884	76.6	0.940	8.7	0.114
20	1.855	71.7	1.001	10.0	0.140
21	1.957	90.6	0.958	9.1	0.100
22	1.784	60.8	1.003	10.1	0.166
23	1.925	84.2	0.941	8.7	0.104
24	1.948	88.8	0.948	8.9	0.100
25	1.904	80.1	0.928	8.5	0.106
26	1.714	51.7	0.980	9.6	0.185
27	1.972	93.7	0.991	9.8	0.105
28	1.905	80.3	0.937	8.6	0.108
29	1.905	80.4	0.925	8.4	0.105
30	1.829	67.4	0.990	9.8	0.145
31	1.760	57.5	0.992	9.8	0.171
32	1.921	83.4	0.946	8.8	0.106
33	1.885	76.7	0.976	9.5	0.123
34	1.760	57.5	0.995	9.9	0.172
35	1.823	66.5	0.906	8.0	0.121
36	1.882	76.2	0.961	9.1	0.120
37	1.891	77.7	1.061	11.5	0.148
38	2.154	142.5	1.034	10.8	0.076
39	1.981	95.7	0.985	9.7	0.101
40	1.947	88.6	0.982	9.6	0.108
41	1.836	68.6	1.058	11.4	0.167
42	1.931	85.4	0.982	9.6	0.112
43	1.904	80.1	1.040	11.0	0.137
44	1.926	84.4	1.000	10.0	0.118
45	1.950	89.2	0.989	9.8	0.109
46	2.182	151.9	1.045	11.1	0.073

APPENDIX XII – Continued

E1	Log $C_{w,floc}$ *1.886*±4.93%	10^	Log $C_{s,floc}$ *0.991*±4.93%	10^	Kd
Trial#					
47	1.865	73.3	1.003	10.1	0.137
48	1.820	66.1	0.991	9.8	0.148
49	1.921	83.4	1.035	10.8	0.130
50	1.747	55.8	0.965	9.2	0.165
51	1.863	73.0	1.004	10.1	0.138
52	1.884	76.6	1.056	11.4	0.149
53	1.963	91.9	0.966	9.2	0.101
54	1.742	55.2	1.013	10.3	0.186
55	1.887	77.1	0.912	8.2	0.106
56	1.920	83.2	1.003	10.1	0.121
57	1.987	97.0	1.075	11.9	0.123
58	1.922	83.5	0.947	8.9	0.106
59	1.851	70.9	0.949	8.9	0.125
60	1.852	71.1	1.028	10.7	0.150
61	1.916	82.5	1.013	10.3	0.125
62	1.923	83.8	0.922	8.4	0.100
63	1.868	73.7	0.907	8.1	0.110
64	2.012	102.7	1.013	10.3	0.100
65	1.910	81.2	0.993	9.8	0.121
66	2.037	108.8	0.976	9.5	0.087
67	1.870	74.2	0.968	9.3	0.125
68	1.914	82.1	0.957	9.1	0.110
69	2.072	118.1	1.022	10.5	0.089
70	1.786	61.1	0.995	9.9	0.162
71	1.874	74.9	1.010	10.2	0.137
72	1.787	61.3	0.960	9.1	0.149
73	1.935	86.2	1.002	10.0	0.117
74	1.736	54.4	0.993	9.8	0.181
75	1.894	78.4	0.970	9.3	0.119
76	1.787	61.2	1.001	10.0	0.164
77	1.941	87.4	1.014	10.3	0.118
78	1.900	79.4	0.985	9.7	0.122
79	1.867	73.7	1.012	10.3	0.140
80	2.018	104.3	1.044	11.1	0.106
81	1.790	61.6	1.086	12.2	0.198
82	1.952	89.6	0.954	9.0	0.101
83	1.720	52.5	0.943	8.8	0.167
84	1.870	74.1	0.989	9.7	0.132
85	1.865	73.2	0.925	8.4	0.115
86	1.766	58.3	0.951	8.9	0.153
87	1.858	72.1	1.003	10.1	0.140
88	1.842	69.5	1.125	13.3	0.192
89	1.784	60.8	0.923	8.4	0.138
90	1.850	70.8	0.990	9.8	0.138
91	1.897	78.8	0.972	9.4	0.119
92	1.870	74.2	1.049	11.2	0.151
93	1.991	98.0	0.975	9.4	0.096

APPENDIX XII – Continued

E1	Log $C_{w,floc}$	10^	Log $C_{s,floc}$	10^	Kd
	1.886±4.93%		0.991±4.93%		
Trial#					
94	1.786	61.1	1.032	10.8	0.176
95	1.863	72.9	1.026	10.6	0.146
96	1.874	74.8	1.040	11.0	0.147
97	1.834	68.3	1.059	11.4	0.168
98	2.015	103.6	0.998	10.0	0.096
99	1.951	89.4	0.955	9.0	0.101
100	2.033	107.9	0.988	9.7	0.090

(Continued for 4800 more runs not shown)

E1	Log $C_{w,floc}$	10^	Log $C_{s,floc}$	10^	Kd
4900	2.002	100.5	0.982	9.6	0.096
4901	1.840	69.2	0.905	8.0	0.116
4902	2.012	102.9	0.910	8.1	0.079
4903	1.780	60.3	1.049	11.2	0.186
4904	1.810	64.6	0.923	8.4	0.130
4905	2.002	100.4	1.077	11.9	0.119
4906	1.842	69.4	1.004	10.1	0.145
4907	1.832	68.0	0.955	9.0	0.133
4908	1.797	62.6	0.895	7.9	0.125
4909	1.834	68.2	0.941	8.7	0.128
4910	1.938	86.6	0.940	8.7	0.101
4911	1.829	67.5	1.031	10.7	0.159
4912	1.746	55.7	0.943	8.8	0.157
4913	1.974	94.1	1.090	12.3	0.131
4914	2.005	101.1	1.025	10.6	0.105
4915	1.919	82.9	0.956	9.0	0.109
4916	2.032	107.7	0.974	9.4	0.087
4917	1.977	94.9	1.030	10.7	0.113
4918	1.779	60.2	0.917	8.3	0.137
4919	1.920	83.1	0.949	8.9	0.107
4920	1.912	81.7	1.001	10.0	0.123
4921	1.930	85.2	1.009	10.2	0.120
4922	1.860	72.5	1.003	10.1	0.139
4923	1.863	73.0	0.983	9.6	0.132
4924	1.958	90.7	0.883	7.6	0.084
4925	1.885	76.7	1.092	12.4	0.161
4926	1.591	39.0	0.985	9.7	0.248
4927	1.869	74.0	1.051	11.2	0.152
4928	1.783	60.7	1.001	10.0	0.165
4929	1.769	58.7	0.964	9.2	0.157
4930	1.850	70.8	1.057	11.4	0.161
4931	1.864	73.2	0.904	8.0	0.109
4932	1.902	79.7	0.994	9.9	0.124
4933	1.864	73.1	0.988	9.7	0.133

APPENDIX XII – Continued

E1	**Log $C_{w,floc}$** *1.886*±4.93%	**10^**	**Log $C_{s,floc}$** *0.991*±4.93%	**10^**	**Kd**
Trial#					
4934	1.873	74.6	1.069	11.7	0.157
4935	1.928	84.6	0.980	9.5	0.113
4936	1.872	74.5	0.973	9.4	0.126
4937	1.957	90.7	0.916	8.2	0.091
4938	1.837	68.8	0.951	8.9	0.130
4939	1.821	66.3	1.030	10.7	0.162
4940	1.741	55.1	0.863	7.3	0.133
4941	1.945	88.0	1.064	11.6	0.132
4942	1.913	81.9	0.993	9.8	0.120
4943	1.981	95.6	1.046	11.1	0.116
4944	1.813	65.0	0.981	9.6	0.147
4945	1.869	74.0	1.014	10.3	0.140
4946	1.984	96.3	0.974	9.4	0.098
4947	1.986	96.7	1.047	11.1	0.115
4948	1.727	53.3	0.973	9.4	0.176
4949	1.808	64.3	0.965	9.2	0.144
4950	1.808	64.3	0.988	9.7	0.151
4951	1.888	77.3	1.087	12.2	0.158
4952	2.080	120.1	1.017	10.4	0.087
4953	1.980	95.5	0.971	9.4	0.098
4954	1.878	75.6	1.084	12.1	0.160
4955	1.842	69.4	1.089	12.3	0.177
4956	1.778	60.0	0.931	8.5	0.142
4957	1.930	85.1	0.952	9.0	0.105
4958	1.875	75.0	0.935	8.6	0.115
4959	1.775	59.6	0.934	8.6	0.144
4960	1.937	86.5	0.942	8.8	0.101
4961	1.787	61.3	1.020	10.5	0.171
4962	1.965	92.3	0.964	9.2	0.100
4963	1.917	82.7	0.985	9.7	0.117
4964	1.776	59.7	1.056	11.4	0.191
4965	1.705	50.7	1.006	10.1	0.200
4966	2.002	100.4	1.119	13.1	0.131
4967	2.031	107.3	1.008	10.2	0.095
4968	1.702	50.4	0.922	8.4	0.166
4969	1.740	55.0	0.941	8.7	0.159
4970	1.952	89.6	1.028	10.7	0.119
4971	1.961	91.3	1.006	10.2	0.111
4972	1.775	59.6	1.005	10.1	0.170
4973	2.017	103.9	0.987	9.7	0.093
4974	1.919	82.9	1.020	10.5	0.126
4975	1.784	60.8	0.936	8.6	0.142
4976	1.748	56.0	1.113	13.0	0.232
4977	1.868	73.8	1.042	11.0	0.149
4978	1.760	57.6	0.967	9.3	0.161
4979	1.976	94.6	0.953	9.0	0.095
4980	1.777	59.8	1.120	13.2	0.220

APPENDIX XII – Continued

E1	**Log $C_{w,floc}$**	**10^**	**Log $C_{s,floc}$**	**10^**	**Kd**
	1.886±4.93%		*0.991*±4.93%		
Trial#					
4981	2.018	104.3	0.997	9.9	0.095
4982	1.841	69.4	0.998	10.0	0.144
4983	1.998	99.5	0.890	7.8	0.078
4984	2.056	113.8	0.952	9.0	0.079
4985	1.945	88.2	0.972	9.4	0.106
4986	1.917	82.6	1.102	12.6	0.153
4987	1.777	59.9	1.057	11.4	0.190
4988	1.847	70.3	0.934	8.6	0.122
4989	1.909	81.2	0.985	9.7	0.119
4990	1.922	83.5	0.991	9.8	0.117
4991	1.946	88.4	0.929	8.5	0.096
4992	2.021	104.9	1.082	12.1	0.115
4993	2.000	100.1	1.090	12.3	0.123
4994	1.817	65.6	1.001	10.0	0.153
4995	1.927	84.5	0.850	7.1	0.084
4996	1.763	57.9	0.964	9.2	0.159
4997	1.962	91.6	1.005	10.1	0.110
4998	1.924	84.0	0.897	7.9	0.094
4999	1.685	48.4	0.968	9.3	0.192
5000	2.048	111.6	0.955	9.0	0.081
					0.131
				%16	0.100
				%84	0.161
				SD	0.032
				Skewness	0.762

At the beginning of the table, Log C columns represent random number generation of a population with mean of he underlined value and error % indicated with ±, in 2nd row of table. At the end of the table %16 and %84 indicate typical ±1 SD percentile (theoretically encompassaing 84%-16% = 68% of the measures). Skewness is a measure of how asymmetrical the distribution is.

$$\mathbf{K_d = 0.13 \pm 0.03}$$

APPENDIX XIII – Wastewater quality, operational and chemical contaminants data

Table A4-3 - Wastewater quality and process data used in calculations.

Day	*Q^a* *(MLD)*	*Q_{waste}* *(MLD)*	*TSS_{raw}* *(mg/L)*	*$TSS_{primary}{}^a$* *(mg/L)*	*$MLVSS^b$* *(mg/L))*	*$TSS_{secondary}{}^a$* *(mg/L)*	*$BOD_{primary}$* *(mg/L)*	*$BOD_{secondary}$* *(mg/L)*
Monday	262.1	1.49	276	68	2460	2.1	107	2.6
Tuesday	256.2	1.49	312	56	2850	2.1	82	2.1
Wednesday	311.5	1.40	316	68	2620	2.7	68	6.0
Thursday	292.3	1.28	212	55	2750	3.0	73	3.2
Friday	269.1	1.29	280	50	3000	4.1	76	2.5
Saturday	239.8	1.28	208	53	3200	2.7	67	2.5
Sunday	254.3	1.28	220	54	3300	2.5	76	2.6

[a] Goldbar Wastewater Quality Laboratory; [b] Analysis performed by authors using Standard Methods for the analysis of water and wastewaters.

Table A4-4 - Concentration of chemical species used for calculations of data summarized in the main body.

Compound *Day*	*Raw* *Diss / Ads* *(ng/L) / (μg/kg)*	*PE* *Diss / Ads* *(ng/L) / (μg/kg)*	*Sludge* *(μg/kg)*	*FECp* *(ng/L)*	*FEC* *(ng/L)*
RYA - total					
Monday	33 / 6.2	184 / 20.5	4.5	63	85
Tuesday	55 / ND	60 / ND	4.8	88	93
Wednesday	45 / ND	64 / 3.1	7.9	214	214
Thursday	36 / 2.1	125 / ND	1.1	138	140
Friday	36 / 8.5	95 / 66	2.0	291	295
Saturday	41 / 10.8	102 / 45	2.0	137	336
Sunday	91 / 28.7	123 / 34	3.1	287	290
Conj - total					
Monday	85 / na	81 / na	na	22	23
Tuesday	133 / na	106 / na	na	36	22
Wednesday	32 / na	42 / na	na	19	19
Thursday	50 / na	53 / na	na	22	22
Friday	94 / na	72 / na	na	41	29
Saturday	133 / na	118 / na	na	48	23
Sunday	129 / na	53 / na	na	28	32

APPENDIX XIII (continued)

Compound Day	*Raw* *Diss / Ads* *(ng/L) / (µg/kg)*	*PE* *Diss / Ads* *(ng/L) / (µg/kg)*	*Sludge* *(µg/kg)*	*FECp* *(ng/L)*	*FEC* *(ng/L)*
Estrone (E1)					
Monday	10 / 4.8	27 / 22	11	52	47
Tuesday	22 / 19	42 / 144	7.1	50	37
Wednesday	14 / 3.0	30 / 26[a]	20	51	51
Thursday	28 / 5.7	86 / 9.4	6.4	53	58
Friday	33 / 28	80 / 55	8.6	101	82
Saturday	25 / 24	69 / 25	9.8	110	93
Sunday	31 / 51	64 / 21	6.4	121	84
Estradiol (E2)					
Monday	1.7 / 2.4	1.6 / 162	0.6	15	10
Tuesday	4.0 / 2.8	7.8 / 4930	0.5	10	12
Wednesday	3.6 / 1.9	5.1 / 45[a]	5.6	16	16
Thursday	17 / 3.5	32 / 13	0.5	16	17
Friday	21 / 3.1	28 / 17	0.4	28	26
Saturday	14 / 1610	23 / 16	2.4	37	29
Sunday	18 / 3.9	22 / 16	0.4	34	29
Estriol (E3)					
Monday	0.7 / 7.0	0.7 / 11	0.4	0.6	0.6
Tuesday	32 / 42	1.2 / 17	1.7	0.6	0.6
Wednesday	0.6 / 40	0.6 / 19[a]	5.2	0.3	0.3
Thursday	9.2 / 228	6.0 / 15	3.1	0.5	0.3
Friday	16 / 70	11 / 40	24	3.6	0.5
Saturday	25 / 15	1.8 / 17	0.4	2.7	0.5
Sunday	12 / 54	8.3 / 14	6.8	1.7	0.4
BPA					
Monday	1230 / 2420	723 / 4320	607	246	470
Tuesday	4330 / 3130	4326 / 14800	358	165	923
Wednesday	84 / 643	349 / 16200[a]	159	ND	ND
Thursday	318 / 7540	199 / 12800	281	11	ND
Friday	1380 / 5400	4712 / 27300	456	176	40
Saturday	5170 / 2750	4332 / 16500	986	423	546
Sunday	11500 / 7100	7055 / 21800	260	374	288

APPENDIX XIII (continued)

Compound Day	*Raw* *Diss / Ads* *(ng/L) / (μg/kg)*	*PE* *Diss / Ads* *(ng/L) / (μg/kg)*	*Sludge* *(μg/kg)*	*FECp* *(ng/L)*	*FEC* *(ng/L)*
NP					
Monday	8450 / 16400	6050 / 21500	739	4640	8890
Tuesday	6700 / 13400	7500 / 6450	ND	4930	4620
Wednesday	8670 / 21700	12200 / 19600[a]	na	2480	2480
Thursday	10700 / na	7400 / ND	641	2110	2280
Friday	12400 / ND	11400 / 43100	2040	4920	4280
Saturday	9140 / 21700	12100 / 17300	2110	6410	7830
Sunday	8890 / 19000	10100 / 9560	835	na	na
Testosterone					
Monday	7.5 / ND	0.2 / ND	ND	0.1	0.1
Tuesday	15 / ND	0.2 / ND	ND	0.1	0.1
Wednesday	6.6 / ND	0.1 / ND	ND	0.1	0.1
Thursday	29 / ND	10 / ND	ND	0.1	0.1
Friday	41 / ND	5.4 / ND	ND	0.1	0.1
Saturday	28 / ND	6.5 / ND	ND	0.1	0.1
Sunday	29 / ND	1.2 / ND	ND	0.1	0.1
Progesterone					
Monday	0.7 / ND	0.1 / ND	ND	0.1	0.1
Tuesday	1.9 / ND	0.2 / ND	ND	0.1	0.1
Wednesday	13 / ND	0.1 / ND	ND	0.04	0.04
Thursday	17 / ND	19 / ND	ND	0.04	0.04
Friday	41 / ND	6.1 / ND	ND	0.1	0.1
Saturday	27 / ND	19 / ND	ND	0.1	0.1
Sunday	29 / ND	1.2 / ND	ND	0.1	0.1
Cholesterol					
Monday	43300 / 256000	31000 / 562000	27700	5490	na
Tuesday	44200 / 139000	35400 / 446000	12400	3420	5970
Wednesday	25000 / 222000	21300 / 398000	59500	3250	3250
Thursday	46900 / na	18600 / 186000	16200	1910	2480
Friday	56200 / 159000	30600 / 432000	21800	2780	2380
Saturday	32100 / 233000	23100 / na	na	1590	2340
Sunday	37100 / 217000	49800 / 412000	na	2430	3120

APPENDIX XIII (continued)

Compound Day	*Raw* *Diss / Ads* *(ng/L) / (μg/kg)*	*PE* *Diss / Ads* *(ng/L) / (μg/kg)*	*Sludge* *(μg/kg)*	*FECp* *(ng/L)*	*FEC* *(ng/L)*
Cholestanol					
Monday	2240 / 30400	1570 / 51700	5390	770	na
Tuesday	2250 / 24600	1750 / 61400	3310	515	922
Wednesday	1443 / 31800	1150 / 40200	15100	547	547
Thursday	3350 / na	1530 / 23800	4490	316	437
Friday	3990 / 30900	2585 / 83300	6910	640	539
Saturday	3880 / 46200	2570 / na	na	595	648
Sunday	4330 / 40400	3540 / 70000	na	944	788
Stigmastanol					
Monday	1180 / 23300	694 / 33600	3420	53	na
Tuesday	1200 / 19200	472 / 41200	2530	235	31
Wednesday	584 / 22000	545 / 30400	11000	85	85
Thursday	1538 / na	332 / 14600	3380	61	ND
Friday	2220 / 22200	761 / 55400	5500	165	64
Saturday	1793 / 29700	1010 / na	na	97	385
Sunday	2110 / 27200	1480 / 55300	na	235	369

ND = not detected. [a]Estimated value.

APPENDIX XIV – Percent (%) log SD and multiplicative standard deviation determined for each analyte.

	%Error (log SD)	*/s		%Error (log SD)	*/s
LC-MS/MS(IS)			**GC-HRMS(IS)**		
Total Conjugates	13.86	1.38	Nonylphenol	2.36	1.06
bisphenol A	9.01	1.23	Bisphenol A	2.62	1.06
equilinin	4.01	1.10	DEHP	2.86	1.07
diethylstilbesterol	14.58	1.40	Coprostan-3-one	2.30	1.05
equilinen	5.39	1.13	Cholesterol	2.60	1.06
estrone	4.93	1.12	Cholestanol (Coprostanol)	4.68	1.11
estradiol	2.49	1.06	Desmosterol	5.51	1.14
hydroxyestrone	3.97	1.10	Stigmastanol	5.42	1.13
totarol	na	na	6-Ketocholestanol	33.45	2.16
estriol	15.26	1.42	7-Ketocholesterol	29.00	1.95
ethynylestradiol	3.24	1.08	estradiol-3-benzoate	10.71	1.28
zearalanol	4.53	1.11			
norgesterol	6.36	1.16	**RYA**		
testosterone	5.88	1.14	ASE (solids)	54.8	3.53
progesterone	5.37	1.13	MTBE	22.0	1.66
estradiol-3-benzoate	21.05	1.62	Ethy Ac	12.9	1.35
norethindrone	6.29	1.16	MeOH	47.7	3.00
Total Free Estro/Pro	9.13	1.23	Total	27.6	1.89
LC-MS/MS (Ex)					
Nonylphenol	2.83	1.07			
NP1EO	2.48	1.06			
NP2EO	2.64	1.06			
bisphenol A	9.03	1.23			

IS = internal standards method; Ex = external standard method; RYA = recombinant yeast assay; */ = multiplicative standard deviation.

APPENDIX XV – Ambient conditions, wastewater quality and operational parameters during sampling period.

Date	Ambient Conditions						
(mm/dd/yyyy)	Effluent Temp (°C)	Mean Air Temp (°C)	Total Rain (mm)	Total Snow (cm)	Total Precip (mm)	Snow on Grnd (cm)	Max Gust (km/hr)
11/09/2006	21.4	14.4	0	0	0		37
12/09/2006	21.6	11.8	19	0	19		<31
13/09/2006	20.4	10.3	5.4	0	5.4		48
14/09/2006	20.2	8.2	19	0	19		44
15/09/2006	17.1	5.4	19.2	0	19.2		48
16/09/2006	17.0	3.2	4.8	0	4.8		33
17/09/2006	18.0	3.8	0.2	0	0.2		<31
18/09/2006	19.2	4	0	0	0		<31
19/09/2006	19.4	6.9	0.2	0	0.2		<31
20/09/2006	19.8	7	0	0	0		<31
21/09/2006	19.6	8.2	5.8	0	5.8		35
22/09/2006	18.3	8.1	0	0	0		<31
23/09/2006	19.1	9.2	0	0	0		41
24/09/2006	19.7	12.9	0	0	0		33
25/09/2006	20.0	13.6	0.8	0	0.8		41
26/09/2006	20.1	7.8	0.2	0	0.2		33
27/09/2006	20.1	7.2	0	0	0		<31
28/09/2006	20.2	13.4	2.8	0	2.8		35
29/09/2006	20.6	15.1	0	0	0		<31
30/09/2006	20.6	13	1.8	0	1.8		46
01/10/2006	20.0	8.3	1	0	1		39
02/10/2006	19.8	4.5	0	0	0		<31
03/10/2006	20.1	4.1	0	0	0		<31
04/10/2006	20.3	7.5	0	0	0		<31
05/10/2006	20.2	9	0	0	0		<31
06/10/2006	20.1	4.7	2.8	0	2.8		<31
07/10/2006	19.2	4.5	0	0	0		56
08/10/2006	19.3	3.3	0	0	0		<31
09/10/2006	19.3	5.5	0	0	0		32
10/10/2006	19.5	4.7	2.4	0	2.4		33
11/10/2006	18.6	1.1	0	0	0		<31
12/10/2006	18.8	2	0	0	0		<31
13/10/2006	19.0	2.5	0	0	0		<31
14/10/2006	19.1	4.7	0	0	0		<31
15/10/2006	19.1	2.5	6.8	0.2	7	0	<31
16/10/2006	17.1	0.4	0	5.2	5.2	1	<31
17/10/2006	17.3	-0.9	0	0	0	0	<31
18/10/2006	18.2	-0.4	0.6	0	0.6		<31
19/10/2006	18.3	2.4	2.8	0	2.8		<31
20/10/2006	17.5	2.4	1	0.6	1.6		50
21/10/2006	17.7	-0.1	0.8	0	0.8		<31
22/10/2006	18.0	4.1	0	0	0		<31
23/10/2006	18.3	6.2	0.4	0	0.4		32

APPENDIX XV – Continued

Date	Effluent	Mean Air	Total Rain	Total Snow	Total Precip	Snow on Grnd	Max Gust
24/10/2006	18.4	5.4	0.2	0	0.2		37
25/10/2006	18.4	3.8	0	0	0		<31
26/10/2006	18.3	3.7	1.4	0.4	1.8		44
27/10/2006	18.1	2.4	0	2	2	1	37
28/10/2006	17.3	-1.9	0	8.3	8.3	3	32
29/10/2006	16.4	-6	0	1.8	1.8	9	35
30/10/2006	17.0	-7.9	0	0	0	8	<31
31/10/2006	17.0	-7.9	0	0.2	0.2	7	52
01/11/2006	16.9	-12.1	0	0	0	7	<31
02/11/2006	17.0	-12.9	0	0	0	7	<31
03/11/2006	16.9	-6.6	0	0.4	0.4	7	<31
04/11/2006	17.1	-5.2	0	0	0	7	<31
05/11/2006	17.0	-6.6	0	1.2	0.6	7	<31
06/11/2006	17.0	-5.2	0.4	2.2	2.6	9	<31
07/11/2006	17.1	-2.2	0	0	0	9	<31
08/11/2006	17.0	-4.4	0.6	9.2	9.8	17	<31
09/11/2006	16.8	-14.4	0	0	0	15	<31
10/11/2006	16.7	-6.9	0	0	0	15	35
11/11/2006	16.6	-12.3	0	0.2	0.2	15	<31
12/11/2006	16.4	-12.5	0	0	0	14	<31
13/11/2006	16.4	-7.9	0	6.2	6.2	14	<31
14/11/2006	16.4	-11.1	0	1.4	1.4	21	41
15/11/2006	16.6	-0.9	0	0	0	21	37
16/11/2006	16.7	-5.7	0	0	0	19	52
17/11/2006	16.6	-5.2	0	0	0	17	<31
18/11/2006	16.7	-5.6	0	0	0	17	32
19/11/2006	16.6	0.9	0	0	0	17	46
20/11/2006	16.7	-0.9	0	0	0	16	54
21/11/2006	16.4	-7.9	0	0.2	0.2	14	35
22/11/2006	16.0	-15.8	0	3	3	17	35
23/11/2006	15.9	-17.5	0	7	6.2	16	<31
24/11/2006	15.6	-19.7	0	0.2	0.2	23	46
25/11/2006	15.7	-23.8	0	2.2	2.2	20	<31
26/11/2006	15.5	-26.8	0	4.6	4.6	22	<31
27/11/2006	15.5	-23.3	0	6.2	6.2	29	<31
28/11/2006	15.6	-27.9	0	0	0	25	<31
29/11/2006	15.6	-26.3	0	0	0	25	<31
30/11/2006	15.7	-10.1	0	2.8	2.2	26	39
01/12/2006	15.6	-21.1	0	0.2	0.2	26	46
02/12/2006	15.4	-20.5	0	2.4	2.4	25	<31
03/12/2006	15.7	-6	0	0.8	0.8	27	32
04/12/2006	15.9	-7.6	0	7.6	6.8	23	<31
05/12/2006	16.1	-1.8	0	0.4	0.4	30	52
06/12/2006	15.9	-8.1	0	0	0	29	39
07/12/2006	16.1	-3.9	0	0	0	31	<31
08/12/2006	16.3	-4.6	0	0	0	27	<31
09/12/2006	16.4	-4.6	0	0	0	27	<31
10/12/2006	16.3	-9.8	0	0	0	27	<31

APPENDIX XV – Continued

Date	Effluent	Mean Air	Total Rain	Total Snow	Total Precip	Snow on Grnd	Max Gust
11/12/2006	16.2	-2.6	0	0	0		32
12/12/2006	16.4	-2.7	0	0	0	27	33
13/12/2006	16.3	-7.7	0	0.2	0.2	26	32
14/12/2006	16.3	-6.8	0	0	0	28	<31
15/12/2006	16.3	-3.4	0	0.2	0.2	28	52
16/12/2006	15.9	-12.1	0	0.6	0.4	28	48
17/12/2006	15.7	-15.5	0	0	0	28	<31
18/12/2006	15.9	-6.2	0	0	0	28	<31
19/12/2006	16.2	-2	0	0	0	28	54
20/12/2006	16.0	-7	0	0	0	27	<31
21/12/2006	16.1	-2	0	0	0	27	44
22/12/2006	16.2	-8.8	0	0	0	27	<31
23/12/2006	15.9	-7.1	0	0	0	27	44
24/12/2006	15.6	-7.6	0	0	0	27	48
25/12/2006	15.6	-5.1	0	0	0	25	33
26/12/2006	15.3	-11.7	0	0	0	25	<31
27/12/2006	15.2	-11.7	0	0	0	25	<31
28/12/2006	15.5	-10.1	0	0.8	0.4	26	<31

WWTP - Water Quality & Operational Data I

Date (mm/dd/yyyy)	Flow (MLD) Bypass	Flow (MLD) Inlet	Flow (MLD) Outlet	Flow (MLD) Membrane	BOD (mg/L) PE	BOD (mg/L) FE	BOD (%Red)
11/09/2006	0.0	259.3	259.3	1.0	79	2.4	97.0
12/09/2006	26.7	300.9	274.2	2.1	64	2.6	95.9
13/09/2006	60.9	376.0	315.1	1.2	76	2.5	96.7
14/09/2006	148.6	442.0	293.4	2.6	52	2.4	95.4
15/09/2006	219.2	529.2	310.0	2.7	29	2.0	93.1
16/09/2006	93.9	404.1	310.2	2.7	44	2.0	95.5
17/09/2006	5.3	300.7	295.4	2.6	61	2.3	96.2
18/09/2006	0.0	297.0	297.0	2.5	76	2.3	97.0
19/09/2006	0.0	285.8	285.8	2.4	76	2.5	96.7
20/09/2006	0.0	277.1	277.1	2.4	83	2.5	97.0
21/09/2006	81.7	343.0	261.4	2.4	67	2.2	96.7
22/09/2006	6.8	291.7	284.9	2.4	74	2.4	96.8
23/09/2006	0.0	270.1	270.1	2.3	72	2.0	97.2
24/09/2006	0.0	265.9	265.9	2.3	91	2.0	97.8
25/09/2006	0.0	282.4	282.4	2.2	83		
26/09/2006	0.0	267.9	267.9	2.2	96	2.0	97.9
27/09/2006	0.0	262.5	262.5	2.3	76	2.2	97.1
28/09/2006	0.0	275.6	275.6	2.1	89	2.4	97.3
29/09/2006	0.0	261.0	261.0	2.1	96	2.4	97.5
30/09/2006	7.6	261.5	253.9	2.2			
01/10/2006	6.8	268.1	261.3	2.2	112	2.8	97.5
02/10/2006	0.0	257.9	257.9	2.2	85	2.5	97.1
03/10/2006	0.0	257.9	257.9	2.4	170	2.5	98.5

APPENDIX XV – Continued

Date	Flow (MLD)	Flow (MLD)	Flow (MLD)	Flow (MLD)	BOD (mg/L)	BOD (mg/L)	BOD (%Red)
04/10/2006	0.0	227.1	227.1	2.4	95	2.7	97.2
05/10/2006	0.0	224.0	224.0	2.4	131	2.5	98.1
06/10/2006	14.3	282.1	267.8	2.4	107	2.4	97.8
07/10/2006	0.0	249.0	249.0	2.5	136	2.6	98.1
08/10/2006	0.0	239.2	239.2	2.5	91	2.8	96.9
09/10/2006	0.0	246.1	246.1	2.4	391	3.2	99.2
10/10/2006	25.9	290.2	264.3	2.4		3.4	
11/10/2006	0.0	263.2	263.2	2.5	96	3.0	96.9
12/10/2006	0.0	254.5	254.5	2.4	98	2.0	98.0
13/10/2006	0.0	245.5	245.5	2.4	101	2.0	98.0
14/10/2006	0.0	245.2	245.2	2.4	98	3.0	96.9
15/10/2006	46.8	299.5	252.7	2.4	90	2.0	97.8
16/10/2006	44.3	308.9	264.6	2.5	64	2.0	96.9
17/10/2006	0.0	237.7	237.7	2.3	140	2.2	98.4
18/10/2006	3.4	248.7	245.3	2.4	70	2.0	97.1
19/10/2006	24.7	259.8	235.0	2.2	101	2.0	98.0
20/10/2006	15.9	299.9	284.0	2.2	97	2.0	97.9
21/10/2006	0.0	259.8	259.8	2.2	102	2.0	98.0
22/10/2006	0.0	255.8	255.8	2.1	113	2.0	98.2
23/10/2006	0.0	266.2	266.2	2.2	116	2.3	98.0
24/10/2006	0.0	249.8	249.8	2.3	118	2.0	98.3
25/10/2006	0.0	254.2	254.2	2.3	136	2.2	98.4
26/10/2006	0.0	259.0	259.0	2.2	128	2.7	97.9
27/10/2006	0.0	258.7	258.7	2.4	156	2.6	98.3
28/10/2006	29.2	310.9	281.7	2.4	112	2.3	97.9
29/10/2006	0.0	266.8	266.8	2.3	106	2.9	97.3
30/10/2006	0.0	234.1	234.1	2.5	135	3.0	97.8
31/10/2006	0.0	208.6	208.6	2.4	143	2.0	98.6
01/11/2006	0.0	213.3	213.3	2.5	134	3.0	97.8
02/11/2006	0.0	247.0	247.0	2.7	146	2.2	98.5
03/11/2006	0.0	247.2	247.2	2.5	118	2.2	98.1
04/11/2006	0.0	243.3	243.3	2.6	128	2.7	97.9
05/11/2006	0.0	238.0	238.0	2.6	115	2.1	98.2
06/11/2006	0.0	250.3	250.3	2.6	124	2.0	98.4
07/11/2006	0.0	248.4	248.4	2.5	125	2.0	98.4
08/11/2006	0.0	249.6	249.6	2.5	157	2.5	98.4
09/11/2006	0.0	248.4	248.4	2.5	126	2.3	98.2
10/11/2006	0.0	244.7	244.7	2.5	115	2.1	98.2
11/11/2006	0.0	241.0	241.0	2.6	106	2.3	97.8
12/11/2006	0.0	207.4	207.4	2.5	113	2.8	97.5
13/11/2006	0.0	206.9	206.9	2.6	103	2.6	97.5
14/11/2006	0.0	202.3	202.3	2.5	143	3.1	97.8
15/11/2006	0.0	243.1	243.1	2.6	146	3.0	97.9
16/11/2006	0.0	254.1	254.1	2.1	144	2.1	98.5
17/11/2006	0.0	234.8	234.8	1.8	115	2.3	98.0
18/11/2006	0.0	242.7	242.7	1.7	114	2.0	98.2
19/11/2006	0.0	231.9	231.9	1.9	126	2.5	98.0
20/11/2006	0.0	258.2	258.2	1.7	137	2.9	97.[illegible]

APPENDIX XV – Continued

Date	Flow (MLD)	Flow (MLD)	Flow (MLD)	Flow (MLD)	BOD (mg/L)	BOD (mg/L)	BOD (%Red)
21/11/2006	0.0	250.6	250.6	1.9	121	3.0	97.5
22/11/2006	0.0	241.9	241.9	1.9	136	2.9	97.9
23/11/2006	0.0	241.8	241.8	1.6	128	2.6	98.0
24/11/2006	0.0	245.4	245.4	2.3	148	5.7	96.1
25/11/2006	0.0	204.8	204.8	2.7	125	3.8	97.0
26/11/2006	0.0	204.1	204.1	2.6	128	6.4	95.0
27/11/2006	0.0	209.4	209.4	2.3	135	6.8	95.0
28/11/2006	0.0	240.9	240.9	2.5	135	35.0	74.1
29/11/2006	0.0	238.3	238.3	2.6	243	32.0	86.8
30/11/2006	0.0	240.0	240.0	2.7	142	4.1	97.1
01/12/2006	0.0	242.2	242.2	2.7	134	3.3	97.5
02/12/2006	0.0	237.8	237.8	2.3	153	3.5	97.7
03/12/2006	0.0	243.6	243.6	3.0	116	3.5	97.0
04/12/2006	0.0	222.3	222.3	3.8	134	3.5	97.4
05/12/2006	0.0	249.7	249.7	3.0	136	2.8	97.9
06/12/2006	0.0	244.2	244.2	2.7	132	3.1	97.7
07/12/2006	0.0	248.1	248.1	2.7	135	3.3	97.6
08/12/2006	0.0	235.2	235.2	2.9	132	2.9	97.8
09/12/2006	0.0	243.4	243.4	3.0	119	3.9	96.7
10/12/2006	0.0	219.5	219.5	3.1	154	4.9	96.8
11/12/2006	0.0	226.6	226.6	2.8	147	3.3	97.8
12/12/2006	0.0	244.3	244.3	2.8	150	2.8	98.1
13/12/2006	0.0	242.6	242.6	2.7	136	2.8	97.9
14/12/2006	0.0	247.0	247.0	2.0	149	2.8	98.1
15/12/2006	0.0	221.7	221.7	2.2	115	2.2	98.1
16/12/2006	0.0	220.6	220.6	2.5	142	2.7	98.1
17/12/2006	0.0	211.5	211.5	2.7	125	2.5	98.0
18/12/2006	0.0	219.6	219.6	2.6	140	3.3	97.6
19/12/2006	0.0	240.8	240.8	2.6	146	3.2	97.8
20/12/2006	0.0	241.9	241.9	2.3	138	3.2	97.7
21/12/2006	0.0	218.2	218.2	2.4	169	3.7	97.8
22/12/2006	0.0	213.6	213.6	2.4	137	3.8	97.2
23/12/2006	0.0	212.9	212.9	2.3	156	4.9	96.9
24/12/2006	0.0	209.5	209.5	2.3	142	4.3	97.0
25/12/2006	0.0	210.8	210.8	2.3	130	3.6	97.2
26/12/2006	0.0	193.7	193.7	2.3	135	3.7	97.3
27/12/2006	0.0	220.8	220.8	2.6	132	3.3	97.5
28/12/2006	0.0	211.8	211.8	2.6	172	4.2	97.6

WWTP - Water Quality & Operational Data II

Date (mm/dd/yyyy)	TSS (mg/L) PE	TSS (mg/L) FE	TSS (%Red)	NH3-N (mg/L) PE	NH3-N (mg/L) FE	NH3-N (%Red)	Oxidized N (mg/L) FE	SRT (days)
11/09/2006	79	0.9	98.9		0.063			6.42
12/09/2006	64	2.7	95.8	20.2	0.059	99.7	3.69	6.63
13/09/2006	76	2.7	96.4		0.123			6.87

APPENDIX XV – Continued

Date	TSS (mg/L)	TSS (mg/L)	TSS (%Red)	NH3-N (mg/L)	NH3-N (mg/L)	NH3-N (%Red)	Oxidized N (mg/L)	SRT (days)
14/09/2006	52	2.9	94.4		0.114		3.47	7.27
15/09/2006	29	2.4	91.7		0.076			7.31
16/09/2006	44	2.4	94.5		0.115			7.20
17/09/2006	61	2	96.7		0.706		6.56	7.06
18/09/2006	76	3.5	95.4		0.874			6.97
19/09/2006	76	2.5	96.7	31.8	1.31	95.9	6.33	7.18
20/09/2006	83	2.7	96.7		1.29			7.01
21/09/2006	67	2.3	96.6	28.2	0.573	98.0	6.23	6.87
22/09/2006	74	2.8	96.2		1.05			6.87
23/09/2006	72	2.7	96.3		2.04			6.87
24/09/2006	91	2.3	97.5		1.04		9.55	6.87
25/09/2006	83	2.3	97.2	27.5	0.253	99.1		6.88
26/09/2006	96	2.5	97.4		0.175		5.81	6.89
27/09/2006	76	2.6	96.6		0.148			7.09
28/09/2006	89	2.6	97.1		0.166		7.09	7.19
29/09/2006	96	2.8	97.1		0.169			7.14
30/09/2006		3			0.423			7.24
01/10/2006	112	2.8	97.5	27.8	0.531	98.1	7.74	7.35
02/10/2006	85	2.4	97.2		0.443			7.41
03/10/2006	170	2.7	98.4		0.225		5.86	7.67
04/10/2006	95	2.6	97.3		0.192			7.77
05/10/2006	131	3.4	97.4		0.245		7.82	7.61
06/10/2006	107	2.6	97.6		0.544			7.53
07/10/2006	136	3.1	97.7		1.12			8.08
08/10/2006	91	3.2	96.5	24.3	1.27	94.8	9.19	11.03
09/10/2006	391	3.4	99.1		1.41			8.85
10/10/2006		2.9			0.448		6.34	8.82
11/10/2006	96	3.6	96.3		0.494			8.87
12/10/2006	98	3.7	96.2		0.316		8.58	8.49
13/10/2006	101	3.6	96.4		0.254			8.47
14/10/2006	98	2.7	97.2		0.17			8.23
15/10/2006	90	2.3	97.4		0.398		8.16	8.30
16/10/2006	64	2.9	95.5		0.149			8.01
17/10/2006	140	3.2	97.7	29.3	0.297	99.0	7.28	8.00
18/10/2006	70	5.6	92.0		0.325			7.90
19/10/2006	101	2.8	97.2		0.401		7.89	7.79
20/10/2006	97	2.5	97.4		0.197			8.24
21/10/2006	102	2.7	97.4		1.19			7.83
22/10/2006	113	2.6	97.7		1.46		9.73	7.70
23/10/2006	116	3.6	96.9		1.27			7.71
24/10/2006	118	2.9	97.5	30.7	0.809	97.4	9.44	7.10
25/10/2006	136	3.2	97.6		0.79			7.76
26/10/2006	128	3.3	97.4		0.806		8.48	7.91
27/10/2006	156	2.5	98.4		0.745			7.66
28/10/2006	112	3.2	97.1		0.906			8.93
29/10/2006	106	3.2	97.0		1.73		7.89	8.14
30/10/2006	135	2.9	97.9		2.48			7.90
31/10/2006	143	2.5	98.3	36.5	1.65	95.5	6.4	7.97

APPENDIX XV – Continued

Date	TSS (mg/L)	TSS (mg/L)	TSS (%Red)	NH3-N (mg/L)	NH3-N (mg/L)	NH3-N (%Red)	Oxidized N (mg/L)	SRT (days)
01/11/2006	134	2.8	97.9		1.73			8.45
02/11/2006	146	2.6	98.2		2.54		6.95	7.22
03/11/2006	118	3.9	96.7		2.57			7.22
04/11/2006	128	3.5	97.3		3.28			7.65
05/11/2006	115	2.9	97.5		3.78		8.12	7.49
06/11/2006	124	3.2	97.4		2.86			7.22
07/11/2006	125	3.6	97.1	27.4	2.34	91.5		7.18
08/11/2006	157	2.5	98.4		2.29			7.36
09/11/2006	126	3.7	97.1		2.54		5.1	7.59
10/11/2006	115	3	97.4		2.95			8.75
11/11/2006	106	2.7	97.5		3.71			8.40
12/11/2006	113	2.9	97.4		3.86			8.40
13/11/2006	103	3.7	96.4	24.1	3.57	85.2	5.75	8.40
14/11/2006	143	3.2	97.8		2.41			8.40
15/11/2006	146	3.5	97.6		3.27			10.66
16/11/2006	144	4	97.2		3.39		4.75	15.70
17/11/2006	115	2.8	97.6		3.27			10.75
18/11/2006	114	2.9	97.5		5.22			11.33
19/11/2006	126	3.3	97.4		6.95		7.46	10.47
20/11/2006	137	4.9	96.4		6.72			12.30
21/11/2006	121	4.3	96.4	29.8	3.73	87.5	5.26	17.94
22/11/2006	136	3.8	97.2		3.07			19.00
23/11/2006	128	3.9	97.0		1.82		4.43	15.81
24/11/2006	148	12	91.9		1.53			11.72
25/11/2006	125	4.1	96.7		2.19			7.22
26/11/2006	128	15	88.3		2.91		6.08	8.87
27/11/2006	135	22.7	83.2		3.54			8.18
28/11/2006	135	9.2	93.2	28.1	3.6	87.2	3.21	8.92
29/11/2006	243	12	95.1		6.47			8.44
30/11/2006	142	4.4	96.9		4.58		4.03	8.98
01/12/2006	134	5.3	96.0		4.68			8.47
02/12/2006	153	6.3	95.9		6.03			8.12
03/12/2006	116	4.3	96.3		7.04		5.89	8.13
04/12/2006	134	5.3	96.0		5.59			8.13
05/12/2006	136	3.5	97.4	30.9	0.912	97.0	8.1	7.81
06/12/2006	132	4	97.0		3.59			7.78
07/12/2006	135	4.4	96.7		3.05		8.97	7.48
08/12/2006	132	4.5	96.6		3.25			7.61
09/12/2006	119	6.7	94.4		3.83			7.92
10/12/2006	154	10	93.5		4.26		9.06	8.61
11/12/2006	147	4.7	96.8		5.24			6.58
12/12/2006	150	3.3	97.8	36.0	4.41	87.8	9.17	6.68
13/12/2006	136	3.6	97.4		3.46			6.84
14/12/2006	149	4.1	97.2		2.32		8.54	7.02
15/12/2006	115	4.9	95.7		2.19			7.40
16/12/2006	142	3.3	97.7		2.38			7.62
17/12/2006	125	3.2	97.4		3.83		9.29	7.38
18/12/2006	140	4.2	97.0		4.74			7.38

APPENDIX XV – Continued

Date	TSS (mg/L)	TSS (mg/L)	TSS (%Red)	NH3-N (mg/L)	NH3-N (mg/L)	NH3-N (%Red)	Oxidized N (mg/L)	SRT (days)
19/12/2006	146	4.3	97.1	37.4	4.07	89.1	9.1	7.42
20/12/2006	138	4.4	96.8		5.02			7.35
21/12/2006	169	5.4	96.8		5.18		9.8	6.89
22/12/2006	137	5.1	96.3		3.99			6.74
23/12/2006	156	4.3	97.2		3.19			6.46
24/12/2006	142	7.2	94.9		3.53		9.33	6.47
25/12/2006	130	4.6	96.5		2.25			6.47
26/12/2006	135	5.3	96.1	45.4	3.04	93.3	13	6.41
27/12/2006	132	5.5	95.8		3.92			6.31
28/12/2006	172	4.8	97.2		2.18		9.45	7.00

Date	**RYA – Final Effluent**				
	Well#1 1:4 dil	**Well#2 1:12 dil**	**Well#3 1:24 dil**	**+ control 1:12 dil**	**E2-Eq (ng/L)**
(mm/dd/yyyy)					
11/09/2006	**1.19**	**1.31**	**1.37**	**2.65**	**66.1**
12/09/2006					
13/09/2006					
14/09/2006	**1.18**	**1.33**	**1.36**	**2.77**	**76.4**
15/09/2006					
16/09/2006					
17/09/2006					
18/09/2006	**1.95**	**1.58**	**1.91**	**4.49**	**143.1**
19/09/2006					
20/09/2006					
21/09/2006	**1.25**	**1.25**	**1.35**	**2.87**	**30.2**
22/09/2006					
23/09/2006					
24/09/2006					
25/09/2006	**1.88**	**1.43**	**1.98**	**3.94**	**106.2**
26/09/2006					
27/09/2006					
28/09/2006	**1.61**	**1.72**	**2.03**	**4.11**	**174.1**
29/09/2006					
30/09/2006					
01/10/2006					
02/10/2006	**1.18**	**1.33**	**1.37**	**2.83**	**76.6**
03/10/2006					
04/10/2006					
05/10/2006	**1.20**	**1.30**	**1.36**	**2.75**	**63.6**
06/10/2006					
07/10/2006					
08/10/2006					
09/10/2006	**1.67**	**1.66**	**1.99**	**5.01**	**160.8**
10/10/2006					

APPENDIX XV – Continued

Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)
11/10/2006					
12/10/2006	1.19	1.34	1.38	2.79	77.9
13/10/2006					
14/10/2006					
15/10/2006					
16/10/2006					
17/10/2006	1.20	1.30	1.37	2.75	62.9
18/10/2006					
19/10/2006	1.19	1.31	1.36	2.80	66.1
20/10/2006					
21/10/2006					
22/10/2006					
23/10/2006	1.19	1.29	1.35	2.74	59.4
24/10/2006					
25/10/2006					
26/10/2006					
27/10/2006					
28/10/2006					
29/10/2006					
30/10/2006	1.19	1.28	1.36	2.83	54.4
31/10/2006					
01/11/2006	1.19	1.29	1.23	2.82	58.0
02/11/2006	1.20	1.25	1.34	2.69	35.3
03/11/2006					
04/11/2006					
05/11/2006					
06/11/2006					
07/11/2006					
08/11/2006					
09/11/2006	1.17	1.32	1.33	2.87	73.0
10/11/2006					
11/11/2006					
12/11/2006					
13/11/2006	1.19	1.30	1.36	2.84	63.2
14/11/2006					
15/11/2006					
16/11/2006	1.26	1.35	1.39	2.73	82.0
17/11/2006					
18/11/2006					
19/11/2006					
20/11/2006	1.20	1.29	1.35	2.75	61.2
21/11/2006					
22/11/2006					
23/11/2006	1.25	1.23	1.34	2.73	10.2
24/11/2006					
25/11/2006					
26/11/2006					
27/11/2006					

APPENDIX XV – Continued

Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)
28/11/2006					
29/11/2006					
30/11/2006	1.19	1.27	1.34	2.81	50.0
01/12/2006					
02/12/2006					
03/12/2006					
04/12/2006	1.21	1.30	1.36	2.86	62.1
05/12/2006					
06/12/2006					
07/12/2006	1.21	1.29	1.33	2.88	57.9
08/12/2006					
09/12/2006					
10/12/2006					
11/12/2006	1.19	1.30	1.34	2.86	62.8
12/12/2006					
13/12/2006					
14/12/2006	1.22	1.30	1.78	2.81	64.5
15/12/2006					
16/12/2006					
17/12/2006					
18/12/2006	1.20	1.29	1.34	2.86	57.1
19/12/2006					
20/12/2006					
21/12/2006✓	1.19	1.30	1.35	2.67	64.7
22/12/2006					
23/12/2006					
24/12/2006					
25/12/2006					
26/12/2006	1.24	1.33	1.32	2.80	75.1
27/12/2006					
28/12/2006	1.20	1.30	1.34	2.83	63.1

RYA – Primary Effluents

Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)	%Reduction
11/09/2006	1.25	1.32	1.34	2.85	71.5	8
12/09/2006						
13/09/2006						
14/09/2006	1.20	1.29	1.34	2.98	59.3	-29
15/09/2006						
16/09/2006						
17/09/2006						
18/09/2006	1.26	1.28	1.31	2.98	56.0	-156
19/09/2006						
20/09/2006						

APPENDIX XV – Continued

Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)	%Reduction
21/09/2006	1.21	1.27	1.31	2.92	50.0	40
22/09/2006						
23/09/2006						
24/09/2006						
25/09/2006	1.22	1.25	1.31	2.80	37.8	-181
26/09/2006						
27/09/2006						
28/09/2006	1.22	1.30	1.34	2.82	62.3	-180
29/09/2006						
30/09/2006						
01/10/2006						
02/10/2006	1.22	1.28	1.34	2.68	52.4	-46
03/10/2006						
04/10/2006						
05/10/2006	1.23	1.26	1.31	2.81	41.3	-54
06/10/2006						
07/10/2006						
08/10/2006						
09/10/2006	1.24	1.39	1.39	2.78	95.4	-69
10/10/2006						
11/10/2006						
12/10/2006	1.25	1.31	1.34	2.94	69.6	-12
13/10/2006						
14/10/2006						
15/10/2006						
16/10/2006						
17/10/2006	1.22	1.28	1.37	2.85	55.2	-14
18/10/2006						
19/10/2006	1.23	1.27	1.31	2.83	48.3	-37
20/10/2006						
21/10/2006						
22/10/2006						
23/10/2006	1.27	1.29	1.48	2.76	58.7	-1
24/10/2006						
25/10/2006						
26/10/2006						
27/10/2006						
28/10/2006						
29/10/2006						
30/10/2006	1.26	1.27	1.32	2.59	51.4	-6
31/10/2006						
01/11/2006	1.30	1.32	1.35	2.83	70.8	18
02/11/2006	1.31	1.33	1.34	2.53	74.5	53
03/11/2006						
04/11/2006						
05/11/2006						
06/11/2006						
07/11/2006						

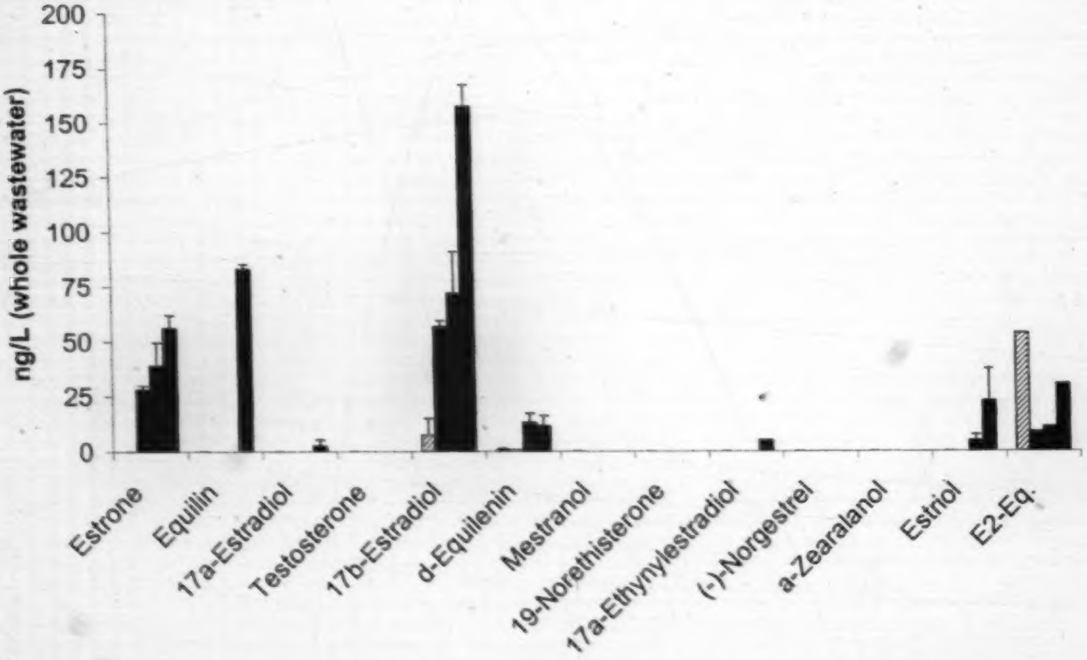
APPENDIX XV – Continued

Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)	%Reduction
08/11/2006						
09/11/2006	1.30	1.12	1.32	2.55	21.8	-234
10/11/2006						
11/11/2006						
12/11/2006						
13/11/2006	1.34	1.29	1.32	2.76	57.0	-11
14/11/2006						
15/11/2006						
16/11/2006	1.34	1.30	1.33	2.72	61.9	-32
17/11/2006						
18/11/2006						
19/11/2006						
20/11/2006	1.31	1.29	1.33	2.83	58.9	-4
21/11/2006						
22/11/2006						
23/11/2006	1.33	1.27	1.42	1.28	25.2	60
24/11/2006						
25/11/2006						
26/11/2006						
27/11/2006						
28/11/2006						
29/11/2006						
30/11/2006	1.31	1.30	1.34	2.84	65.3	23
01/12/2006						
02/12/2006						
03/12/2006						
04/12/2006	1.36	1.34	1.33	2.83	78.0	20
05/12/2006						
06/12/2006						
07/12/2006	1.30	1.35	1.41	2.56	83.8	31
08/12/2006						
09/12/2006						
10/12/2006						
11/12/2006	1.34	1.33	1.36	2.71	76.6	18
12/12/2006						
13/12/2006						
14/12/2006	1.34	1.32	1.34	2.60	70.1	8
15/12/2006						
16/12/2006						
17/12/2006						
18/12/2006	1.31	1.31	1.34	2.86	67.2	15
19/12/2006						
20/12/2006						
21/12/2006	1.31	1.30	1.34	2.52	65.6	1
22/12/2006						
23/12/2006						
24/12/2006						
25/12/2006						

APPENDIX XV – Continued

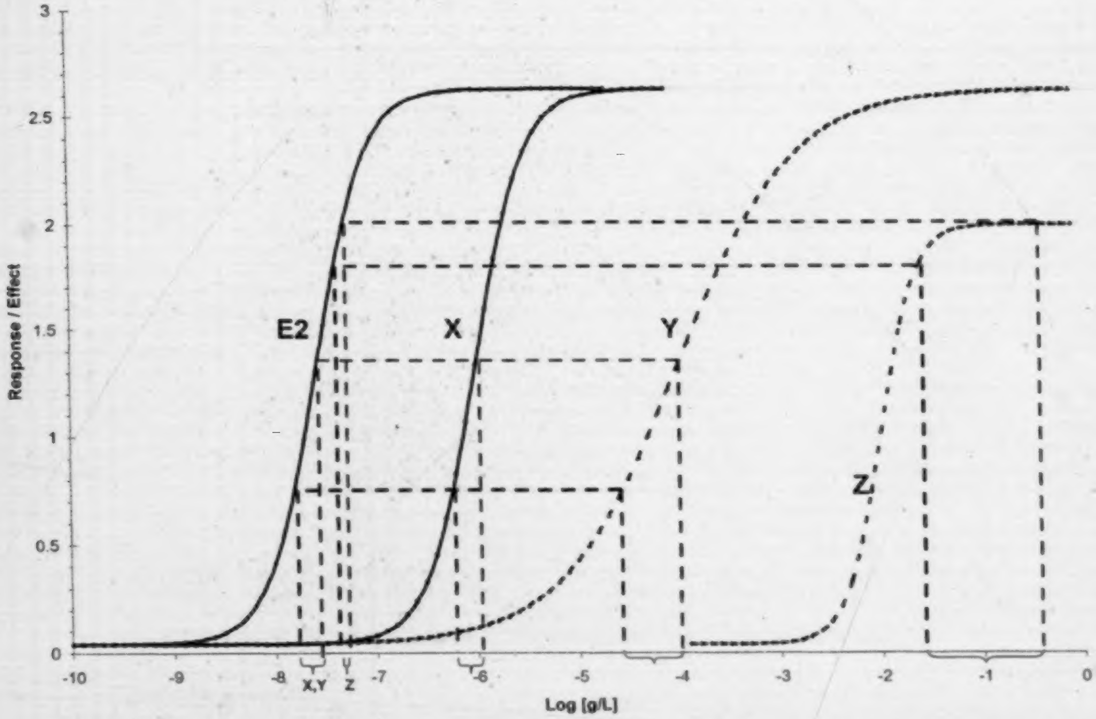
Date	Well#1 1:4 dil	Well#2 1:12 dil	Well#3 1:24 dil	+ control 1:12 dil	E2-Eq (ng/L)	%Reduction
26/12/2006	1.18	1.18	1.63	2.48	440.8	83
27/12/2006						
28/12/2006	1.41	1.30	1.36	2.56	62.3	-1

APPENDIX XVI – Average (n=3) steroidal estrogen concentration and RYA measured estradiol equivalents (E2-Eq.) in three temporal effluent samples from an aerated lagoon.



NOTE: Effluent (soild bars) collected chronologically from left to right and influent sample (horizontal line bar) collected on last sampling date only from site E, Chapter 2, Table 2-1 during sampling period with effluent temperatures of 4, 8, 15°C, respectively. Error bars are standard deviations from triplicate extraction and analysis of each sample.

APPENDIX XVII - Considerations for the interpretation of estrogenic equivalence (EEq) for xenoestrogens and environmental mixtures in this work.



From left to right, the first DRC is for E2, the second is for compound X which has an identical hillslope and maximum response as E2, the third is for compound Y which has a different hillslope but same maximum, and the last chemical Z has a lower maximum response than that of E2.

When an EEQ (in ng E2 per liter) is calculated for a certain test chemical or mixture (i.e. X, Y or Z) using the estrogenic equivalency factor (EEF) calculated as the EC$_{50}$ for E2 / EC$_{50}$ for test substance, the assumption is that the DRC of the test chemical or mixture has the same slope and produces the same maximal response as E2. We see that for test chemical/mixture X, the DRC fulfills these assumptions and the EEF is valid for all concentrations of X, since the ratio of concentrations producing equal response is constant a every point of the curve. For test chemical/mixture Y however, this is not the case and we see that the EEF is not preserved which would lead to a situation, where the chemical EEq (as determined from the EEF determined at EC$_{50}$) would be different than the actual *in vitro* measured EEq for all concentrations not equal to the EC$_{50}$. For chemical/mixture Z, the maximum occurs at a lower response that for E2, resulting in a loss of proportionality as once the chemical/mixture Z is present at maximal responses, any new addition of this chemical will not result in an increase in response and the calculated EEq would not change by much even though the concentration of Z was still increasing. In order to minimize these types of errors in this present thesis work, wherever possible samples were diluted to obtain a mid-range response which was then used for the calculation of EEq.